

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of:
James P. Elia

Serial No.: 09/836,750

Filed: April 17, 2001

For: METHOD FOR GROWING
MUSCLE IN A HUMAN
HEART



Group Art Unit: 1647

Examiner: Daniel C. Gamett

CERTIFICATE OF MAILING

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APPELLANT'S APPEAL BRIEF

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REAL PARTY IN INTEREST

The real parties in interest in the instant appeal are Assignees, Dental Marketing Specialists, Inc., an Arizona corporation, 9377 E. Bell Road, Suite 385 Scottsdale, Arizona 85260, and Jerry W. Bains and Salee C. Bains Irrevocable Trust, 9013 Red Lawrence Drive, Carefree, Arizona 85377. Subsequent to the assignment recordal for the instant application, the address of Dental Marketing Specialists, Inc., changed to 7364 East Crimson Sky Trail, Scottsdale, Arizona 85262. Also, subsequent to the assignment recordal for the instant application, the address of Jerry W. Bains and Salee C. Bains Irrevocable Trust, changed to 39096 N. 102nd Way, Scottsdale, Arizona 85262.

RELATED APPEALS AND INTERFERENCES

There are three other appeal proceedings known to Appellant's legal representatives, which may be related to, directly affect, or may have a bearing upon the Board's decision in the pending appeal. Such appeal proceedings are:

1. Co-pending Serial No. 09/794,456, filed February 27, 2001, in which Appellant's Brief was filed with the Patent and Trademark Office (hereinafter "PTO") on February 6, 2009;
2. Co-pending Serial No. 10/179,589, filed June 25, 2002, in which a Notice of Appeal was filed with the PTO on February 19, 2009; and
3. Co-pending Serial No. 09/064,000 filed April 21, 1998, in which a Notice of Appeal was mailed to the PTO on May 20, 2009.

There are no related interferences or judicial proceedings known to Appellant, Appellants' legal representatives, or Assignee, which may be related to, directly affect, be directly affected by, or may have a bearing on the Board's decision in the pending appeal. The attached Related Appeals and Appendix confirms the above statements.

STATUS OF CLAIMS AND CLAIMS UNDER APPEAL

Claims 1-5 were cancelled in the Preliminary Amendment filed April 17, 2001.

Claims 204, 205, and 237 were cancelled in the Amendment filed February 17, 2004.

Claim 243 was cancelled in the Amendment filed March 2, 2009.

Claims 245, 246, 248, 249, 252, 264-267, 272-279, 286, and 287 were cancelled in the Amendment filed October 15, 2007. Such or similar claims are pending in co-pending applications Serial No. 11/605,153, filed November 28, 2006 and Serial No. 09/794,456, filed February 27, 2001. By cancelling such claims, Appellant has chosen to reduce the number of issues for the instant appeal and does not acquiesce to, or in any way agree with, the correctness of any rejection of these claims in the prosecution of the present application.

Claims 254-256 were cancelled in the Amendment filed November 21, 2005.

Claims 6-235 and 240-242 stand withdrawn, by the PTO, from consideration as being directed to a non-elected invention. As set forth above, claims 204 and 205 were cancelled by Appellant in the Amendment filed February 17, 2004, and thus were incorrectly identified by the PTO as being withdrawn.

In view of the cancellation of the above-identified claims, the correctness of the PTO's rejection in the Office Action dated October 2, 2008, (hereinafter referred to as "the Office Action") of claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 under 35 U.S.C. §112, first paragraph, for lack of enablement, constitutes the sole issue on appeal.

STATUS OF AMENDMENTS

An Amendment correcting a typographical error in claim 239 (which incorrectly identified the dependency of claim 239) was filed on February 21, 2008. No indication was given in the Office Action that such Amendment was entered. Claim 239, as shown in Claims Appendix, indicates the correct dependency.

In addition, an Amendment canceling claim 243 and changing the dependency of claims 244 and 260 to depend upon claims 236 and 244, respectively, was filed March 2, 2009. No indication has been received from the PTO that this Amendment was entered. The Claims Appendix indicates such Amendment.

SUMMARY OF CLAIMED SUBJECT MATTER

Appellant's invention is directed to a method of using well-known compositions (materials), old and well-known administration techniques for such compositions, and equally old and well-known medical apparatus to produce a novel result, i.e., the use of growth factors, including a cell (stem cell), such as bone marrow stem cells ("BMC's"), to grow a new artery and new cardiac muscle in the heart of a human patient and also to growing such new artery and cardiac muscle and repair a dead or damaged portion of a heart. Antecedent bases in the specification for various claim elements are included below.

Appellant's novel contribution to the medical art is defined in the broadest scope in independent generic claim 236 on appeal as comprising a method for growing a new portion of a human heart by placing a growth factor in the body of a human patient and forming a new artery and new cardiac muscle. Claims 238 and 239 cause repair of dead and damaged portions of the heart with the growth of new arteries and cardiac muscle (page 45, lines 17-22; page 46, lines 3-14). A growth factor, as called for by claim 236, broadly encompasses compositions and living organisms, which promote the growth of soft tissue in the body of a patient (page 20, lines 10-14). Appellant's specification on page 21 broadly recites that, "The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle...or by any other desired method." Appellant's invention specifically describes using patient size, vascularity, simplicity of access, ease of exploitation, or any other desired factors in determining the selected area of the patient for administering said growth factor (page 45, lines 1-16). Appellant describes dosages

of growth factors useful for achieving growth of a new artery and achieving heart repair as defined in claims 236, 238, and 239 (page 53, lines 13-19; page 56, lines 7-19; and page 62, lines 1-10) and describes monitoring heart repair by determining blood flow through the new artery by using any readily available commercial device such as ultrasound, angiogram, etc. (page 56, lines 20-25). The specification on page 47 discloses that booster shots of growth factor may be required to repair an organ that is not operating at a desired capacity.

Claims 244 and 271 further limit the invention by specifying that the growth factor of claims 236 and 259, respectively, comprises a cell (page 37, lines 19-26). Claims 247, 268, and 269 directly depend from claims 236, 262, 263, respectively, and further limit the method of said claims 236, 262, and 263, respectively, by reciting that the growth factor is placed in said patient by injection (page 21, line 5; page 45, line 14); and claim 251 depends from and further limits the method of claim 236 by requiring the growth factor be placed in said patient by a carrier (page 21, lines 3-6). Claim 253 depends from claim 236 and requires that the growth factor comprises a gene and a cell (page 46, lines 6-9). Claims 257, 258, 259, and 260 directly depend from and further limit claims 236, 238, 239, and 244, respectively, by requiring that the growth factor is locally placed in the human body (page 21, lines 4-10 and page 46, lines 3-9). Claims 261, 262, and 263 directly depend from and further limit claim 236, 238, and 239, respectively, by requiring that the growth factor involved in the growth of new arteries and new cardiac muscle (claim 261), the repair of dead portions of the heart (claim 262), and the repair of damaged portions of the heart (claim 263) comprises living stem cells harvested from bone marrow (page 40 lines 27 – page 42, line 30). Claims 270 and 271

directly depend from claims 258 and 259, respectively, and require placing a cell into the heart adjacent a dead (claim 270) or damaged (claim 271) portion of the heart causing new arteries and new cardiac muscle to be grown and repair of dead (claim 270) and damaged (claim 271) portions of a human heart to be repaired (pages 45 and 46). Claims 280, 281, and 282 directly depend from claims 236, 238, and 239, respectively, and require calculating blood flow through the newly grown artery (page 56, lines 20-25). Such calculation provides an indication of artery growth. Claims 283, 284, and 285 directly depend from claims 236, 238, and 239, respectively, and require observing the newly grown arteries (page 56, lines 20-25). Such observation provides a description of artery growth. Claims 288-290 depend directly or indirectly from claim 261 and require the placement of stem cells harvested from the patient. The disclosure embracing the subject matter of these claims can be found, for example, in the specification at pages 21, line 4-15; page 32, lines 9-11; page 33, lines 8-10; page 40, line 27 to page 42, line 27; page 44, lines 12 and 13; page 46, lines 3-10; page 47, line 22 to page 48, line 15; Example 19 on page 55, line 14 to page 65, line 25; and Example 36 on page 62.

GROUND OF REJECTION FOR REVIEW ON APPEAL

Appealed claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 stand rejected in the Office Action under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

ARGUMENT

Rejection of Claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 Under 35 U.S.C. §112, first paragraph

Claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285 and 288-290 stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. Appellant responds to the rejection of claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285 and 288-290 in the following two sections, wherein patentability is argued separately in each section.

Rejection of Claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285 Under 35 U.S.C. §112, first paragraph

The PTO rejected appealed claims 236, 238, 239, 244, 247, 250, 251, 253, 257-260, 270, 271, and 280-285 “under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement.” Specifically, the PTO, at pages 2-3, ¶4 of the Office Action, states that:

The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Appellant disagrees that the scope of protection provided by the appealed claims is not adequately enabled by the application disclosure. Appellant intends to, and does hereinafter, argue the patentability of each claim separately, i.e., the patentability of the claims on appeal do not stand or fall together.

It is axiomatic that enablement issues are determined by consideration of an applicant's specification along with knowledge in the art at the time of filing, United States v. Telectronics, 857 F. 2d 778, 785; 8 USPQ 2d 1217, 1223 (Fed. Cir.1988, *cert. denied* 490 U.S. 1946 (1989)). Appellant believes that the instant specification, when considered in view of the knowledge in the art at the time the application was filed, enables one skilled in the medical art to make and use the claimed invention.

Appellant submits that there are three major points to consider when determining whether the instant specification contains a disclosure that would have enabled a skilled person in the medical art to make and use the claimed invention within the purview of the statute. The points are: 1) the content and guidance provided in the specification disclosure; 2) the knowledge in the art at the time the application was filed; and 3) the skill level in the art. When these points are considered, there should be no doubt that Appellant's specification provides an enabling disclosure. The three points are discussed below.

First, as set forth in the **Summary of Claimed Subject Matter** portion of the instant Brief, there is a considerable body of disclosure relating to Appellant's generic invention of repairing organs in human patients, including the heart, by growing new cardiac muscle and a new artery and to elected and non-elected growth factors suitable for effecting such repair and growth. In this regard, Appellant's specification provides a substantial body of disclosure regarding growing and/or replacing organs and/or growing arteries and tissues using well-known compositions, which promote soft tissue growth. The specification describes a class of compositions for promoting soft tissue that broadly and specifically includes genes, nucleic acids, a patient's own cells, universal cells, e.g.,

stem cells, germinal cells (pages 47 and 48), and “enucleated ovum” and “other subunits of a cell” (page 52), which qualify as growth factors and are useful for promoting tissue (organ) growth through differentiation and morphogenesis. The PTO’s selective reading, which ignores Appellant’s broad and specific disclosure relating to non-elected species disclosure, is clearly erroneous under relevant case law. When an applicant elects to prosecute a species following an election requirement, the PTO is not permitted to wear blinders and focus solely upon the elected species while ignoring the scope of enablement provided by the specification as a whole. There should be no doubt that the specification taken as a whole, when properly read and understood by one skilled in the art, meets the statutory requirement for enablement under current law. See In re Anderson, 471 F.2d 1237, 176 USPQ 331, (CCPA 1973) and In re Johnson and Farnham, 558 F.2d 1008, 194 USPQ 187, 195 (CCPA 1977).

That the PTO has failed to review the application disclosure in its entirety is clear from the statement at page 6, lines 1-8 of the February 22, 2006 Office Action for co-pending application Serial No. 09/794,456 filed February 27, 2001, (of record) that:

The claims are being examined to the extent that they read on the elected invention, administration of cells, and thus the generic concept of growth factor is not relevant. [emphasis added].

Second, the PTO has not taken issue with the fact that the administration techniques and administered materials disclosed by Appellant were individually old and well known as of the filing date of the instant patent application. The materials and administration techniques disclosed by Appellant were routinely employed in the medical art, but not in the claimed combination, at the time the instant application was filed.

Appellant's contribution to the medical arts resides in the claimed method of repairing a dead/damaged heart in a human patient by implanting cells and forming cardiac muscle and an artery, thereby causing said heart to be repaired.

The PTO has failed to consider and accord appropriate evidentiary weight pertaining to the state of the prior art at the time of Appellant's invention as exemplified by U.S. Patent No. 5,980,887 to Isner et al. (hereinafter "Isner '887" and of record) and the Asahara et al. February 14, 1997 publication in Science entitled, "Isolation of Putative Progenitor Endothelial Cells for Angiogenesis," (hereinafter "Asahara" and of record), which was cited in Isner '887 and describes therapeutic methods for implanting cells in treating ischemic tissue. Specifically, Isner '887 and Asahara are indicative of contemporary prior art knowledge which employed a limited subpopulation of EC progenitor stem cells isolated from human peripheral blood for promoting capillary growth. Isner '887 and Asahara evidence that those skilled in the art prior to the 1998 filing date of Appellant were aware that EC progenitor cells (stem cells) and DNA encoding VEGF are alternative angiogenesis promoters for treating blood vessel injuries, i.e., ischemic tissue. Isner '887 at column 7, lines 17-23 of the patent, discloses that "any suitable means" can be used to administer stem cells, including intramuscular injection. Additionally, U.S. Patent No. 5,328,470 to Nabel et al. (hereinafter "Nabel" and of record) further evidences that it was known in the art that cells and genes can be either locally or systemically (by injection) administered to human patients to treat organs affected by disease, including ischemic tissue. Although these published works are directed to different inventions than that of Appellants, i.e., employ different cells, and other soft tissue formers to achieve different results, they nevertheless apprise one skilled

in the art of commonly used prior art methods and thus must be taken into consideration by the PTO when determining enablement of the claimed invention under 35 U.S.C. §112, first paragraph.

One skilled in the art reading the instant specification's teaching of using stem cells harvested from the bone marrow or peripheral blood would understand that the claimed invention distinguishes from Nabel, Isner '887, and Asahara by describing using unfractionated (global) bone marrow mononuclear cells for promoting the growth of cardiac muscle and arteries. Moreover, there is no basis in fact for determining administering genes and cells for the treatment of human diseases involving ischemic tissue requires materially different treatment protocols. Appellant's claimed method differs from such existing prior art in regard to the stem cell population, i.e., Appellant's invention requires the transplantation of the entire array of mononuclear cells harvested from bone marrow and achieves the claimed cardiac muscle and artery growth. One skilled in the art being so apprised when reading the instant specification would understand that Appellant has provided sufficient information, i.e., the process steps, ingredients and instrumentation essential to grow an artery and cardiac muscle as set forth in the claims.

Third, the PTO has acknowledged that the level of skill in the art to which the invention pertains is high. Appellant agrees that the skill level is high when it is considered that many years of education, training, and experience are required in the medical field to achieve a high level of skill. Such experience includes the knowledge of prior and contemporary cell therapy practices, including the compositions and methodology used in cell therapy procedures. Cf. United States v. Teletronics, supra.

This is the skill level to which the instant application is addressed and which should be applied by the PTO in determining enablement issues in the instant application.

Once the above-identified relevant materials and administration techniques set forth in Appellant's specification are properly considered in their entirety, Appellant believes that there should be no question that one skilled in the medical art is enabled to make and use the claimed invention. This conclusion is reinforced, as noted above, by the fact that the materials and administration techniques, but not the inventive results, were well known when the instant application was filed. MPEP Section 2164 states that the purpose of the enablement requirement is to describe the claimed invention in such terms to permit one skilled in the art to make and use the invention. Such Section cautions that detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention. MPEP Section 2164.01 states that:

A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F2d. 660, 661, 18 USPQ 2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F2d. 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) cert denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist and Derrick Co.*, 730 F2d. 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

Appellant believes that the above caution is especially relevant to the instant factual situation where the Examiner has conceded that there was a high level of skill in the art at the time the instant application was filed. The PTO has not taken issue with Appellant's position that all the materials, methods, and apparatus needed to practice the invention were well known at the time of the invention. Thus, Appellant submits that it is evident that the instant disclosure clearly enables one skilled in the medical arts to make and/or

use the full scope of the claimed invention without undue experimentation because a reasonable consideration of the three above-delineated factors and the interaction thereof by a skilled person in the medical art leads to the inevitable conclusion that the disclosure is enabling.

The PTO has the burden to establish and support by convincing objective evidence a *prima facie* case of lack of enablement. For reasons set forth below, Appellant believes the PTO has failed to meet such burden.

At the outset, it is noted that the PTO, at page 4, ¶6 of the Office Action states that, for the claims on appeal, the determination of a lack of enablement is focused primarily with respect to the administration of stem cells harvested from bone marrow and that even if claims to such subject matter were enabled, such enablement would not extend to the other claimed methods. It is and always has been Appellant's understanding that generic claim 236 and non-cell claims depending therefrom were only considered by the PTO to the extent they were drawn to the elected invention—cells. Appellant notes that at pages 4 and 5, ¶7 of the Office Action, the PTO questions whether bone marrow stem cells were ever identified in the specification as a “growth factor” as recited in the claims. As pointed out above, the claims drawn to the genus “growth factor” were not the elected invention. The elected invention involves cells and, more specifically, bone marrow comprising cellular components such as stem cells. It is clear from the instant specification at page 20 that growth factors are defined as compositions that promote the growth of soft tissue and include “living organisms,” such as cellular compositions. Moreover, the PTO has acknowledged the fact that cells are, reasonably, living organisms. See page 33, lines 10 and 11, ¶44 of the Office Action in this regard.

Moreover, claims 261-263, 268, and 269 are drawn specifically to stem cells harvested from bone marrow, not to the genus growth factor. The specification teaches that bone marrow stem cells differentiate during morphogenesis into an organ. See pages 42, 47, and 48 of the specification. Page 52 discloses that an enucleated ovum and other subunits of cells qualify as growth factors, i.e., induce the formation of tissues and organs.

The PTO, at the sentence bridging pages 7-8, ¶11 of the Office Action, states that the claims are drawn to “methods of causing formation of an artery that did not previously exist...” The PTO further states that “the issue is that the instant specification does not teach the skilled artisan *how* to manipulate these allegedly old materials and methods to achieve the remarkable effects required by the claims.” Appellant’s specification at pages 54, 56, and 62 clearly defines the claimed term “new artery,” and the scope of the claims is legally determined by this disclosure. It is clear from such disclosure what Appellant intended the term “new artery” to mean, and the claims on appeal must be interpreted accordingly. See Phillips v. AWH., Corp., 415 F. 3d 1303, (Fed. Cir. 2005) in this regard. While Appellant agrees that the state of the art at the time the instant application was filed does not disclose the growth of new arteries, there can be no doubt that post-filing publications of record, including Orlic et al. (hereinafter “Orlic”); Strauer et al. (hereinafter “Strauer”); and Dohmann et al. (hereinafter “Dohmann”) confirm Appellant’s disclosed and claimed results, i.e., heart repair and formation of new cardiac muscle and an artery. Like Appellant, the three above-mentioned post-filing publications employed the entire array of bone marrow cellular components, including stem cells, not an isolated component thereof such as used by Isner ’887 and Asahara.

Orlic describes injecting bone marrow mononuclear stem cells into rats having infarcted hearts and provides autopsy confirmation of the formation of arteries and arterioles (small arteries). Note that Orlic, as well as Dohmann, used the term “smooth muscle actin,” to positively identify the formation of arteries at autopsies. Orlic used enhanced green fluorescent protein to identify the formation of endothelial cells. Unlike capillaries, which are composed of endothelial cells, arteries and arterioles additionally require smooth muscle cells. In this regard, please see Orlic at page 702, left column, top of page where it is stated that:

This allowed us to identify each cardiac cell type, and to recognize endothelial and smooth muscle cells organized in coronary vessels (Fig. 3a-c; see also Supplementary Information). The percentages of new (emphasis added by Appellant) myocytes, endothelial cells, and smooth muscle cells expressing EGFP was 53 +/- 9% (n=7), 44 +/- 6% (n=7) and 49 +/- 7% (n=7), respectively.

From the foregoing, one skilled in the medical arts would have no difficulty in recognizing the Orlic work resulted in growth of new arteries.

Strauer used the term “neovascularization” (page 1913, abstract) to describe the results of the administration of bone marrow mononuclear cells to a heart of a human patient to achieve repair. Obviously, neovascularization includes new artery formation, not merely capillary formation, in view of the impressive improvement in heart function described at page 1917, top of page. Note further at page 1916, right column, paragraph 1 to where it is disclosed that:

... in several animal infarction models it has been shown that: (1) bone marrow hemangioblasts contribute to the formation of new vessels (emphasis added by Appellant); (2) bone marrow hematopoietic stem cells differentiate

into cardio myocytes, endothelium, and smooth muscle cells (emphasis added by Appellant) [ref 8-13...]

Note that “ref 11” of Strauer in the above quotation is the Orlic publication. Being that Strauer and Orlic administered bone marrow mononuclear cells to achieve heart repair, one skilled in the medical art would readily recognize that Strauer also formed new arteries.

Dohmann provides evidence that earlier trials involving implantation of bone marrow mononuclear cells resulted in new artery formation and heart repair via autopsy confirmation on a trial patient. Such trials involved injection (page 2, abstract) of bone marrow mononuclear cells into the heart of a human patient to repair the heart. Note that Figures 3C, D, and E on page 9 of Dohmann indicate that the smooth muscle walls comprise many cell thicknesses of smooth muscle and that smooth muscle actin was identified in the autopsy. As is well known in the medical art, capillaries comprise tubes of one cell thickness. Hence, those skilled in the medical art refer to a capillary wall, not capillary walls.

At page 8, under “Immunocytochemistry Findings,” lines 6-8, Dohmann states:

The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls [emphasis added by Appellant], which had a marked hypertrophy of smooth muscle cells (Figure 3 D).

Dohmann uses the word “new” to describe the formation of blood vessels. See page 11, paragraph 3, line 1 to the bottom of the page in this regard. Inasmuch as Dohmann, along with Orlic and Strauer, describes obtaining endothelial cells and smooth

muscle cells, there can be no doubt that the formed new blood vessels include newly formed arteries because arteries require both endothelial and muscle tissue.

Such disclosure is clearly contrary to the PTO's unsupported assertion that a new artery is not grown. The fact that Appellant, Orlic, Strauer, and Dohmann all administer bone marrow mononuclear cells to achieve heart repair and that all three publications describe new cardiac muscle and artery formation would be readily understood by one skilled in the art as demonstrated above. Regarding the PTO's charge that the specification fails to "teach the skilled artisan how to manipulate" stem cells to achieve the claimed results, one skilled in the art would understand that except for injecting the stem cells into sites or adjacent to sites of ischemic injury, no further "manipulation" is required. Certainly, no manipulation of the injected stem cells was described or required by Orlic, Strauer, and Dohmann. Once the stem cells are implanted, they foci to the ischemic injury site via predetermined genetic pathways wherein differentiation and morphogenesis promotes the growth of new cardiac muscle and arteries. Of particular note is the following statement of William O'Neil found in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, article entitled, "Tissue Engineering and Interventional Cardiology" (of record and attached hereto as Exhibit A). Exhibit A is a complete copy of the above article and is attached because the copy furnished by the PTO is incomplete and does not contain the following quotation:

... in terms of the degree of our ignorance about the basic science in this area. My own feelings is that God – or nature – in His infinite wisdom, is a lot smarter than we will be for a few centuries yet in terms of the cascade of the processes that actually allow a new cell to come in and regenerate.

The above quote highlights that even the highly skilled medical artisan has limitations.

The first paragraph of the statute requires nothing more than objective enablement, and it is of no importance whether such teaching is set forth by use of illustrative examples or by broad terminology. As a general matter, an application disclosure, which contains a teaching of how to make and use the invention in terms which correspond in scope to those used in describing the invention sought to be patented, is considered to be in compliance with the enabling requirement of the statute. In re Marzocchi, 58 CCPA 1069, 439 F.2d 220, 169 USPQ 367, 369-370 (1971). Further, “Section 112 does not require that a specification convince persons skilled in the art that the assertions therein are correct.” [emphasis added]. In re Robins, 429 F.2d 452, 166 USPQ 552 (CCPA, 1970).

When evaluating enablement, it is incumbent upon the PTO to determine what subject matter each claim recites, i.e., the scope of protection sought for each claim. The scope of dependent claims are properly determined with respect to 35 U.S.C. §112, fourth paragraph. See MPEP Section 2164.08. It is clear that the PTO analysis did not treat the subject matter of each claim separately or treat the dependent claims according to statutory mandate.

The PTO states that the underlying fact at issue is whether or not more than routine experimentation would be required to practice the claimed invention and addresses this issue by reference to the guidelines established in In re Wands, 858 F.2d 731, 737, 8 USPQ 2d 1400, 1404 (Fed. Cir.1988). As evidence in support of the non-enablement rejection, the PTO apparently has relied upon Strauer as establishing that a

determination of cell population is critical, citing pages 1916-1917 of the publication. The PTO fails to point to any specific teaching in the record which supports this proposition, and for good reason. Careful review of this publication fails to reveal any teaching that experimentation was required to determine cell population.

At page 8, ¶12 of the Office Action, the PTO stated that nothing in the record indicated that the PTO agreed that the addition of the limitation “forming new arteries” led to a determination of enablement. Appellant never suggested such determination and merely identified the antecedent basis for such term in the specification. In fact, the record indicates that the language was added at the suggestion of the PTO to define over the Murry et al. publication (of record).

At pages 9-10, ¶¶ 13-14 of the Office Action, the PTO takes issue with Appellant’s citation of Nabel as evidencing that methods and apparatus employed by Strauer were well known. Such contention ignores the fact that no experimentation was required by Strauer because old, well known methods and apparatus, such as the off-the-shelf angioplasty technique of Nabel, were used. Appellant cited Nabel to show that experimentation as to administration technique was not required by Strauer. The fact that Nabel failed to use the type of cells necessary for achieving the claimed result, i.e., growing new cardiac muscle and new arteries, does not detract from the fact that such administration was known in the medical art prior to Appellant’s filing date. Appellant relied upon Nabel to provide evidence that angioplasty techniques could be used to deliver therapeutic compositions, such as cells, to the humans prior to Strauer’s published work as well as Appellant’s filing date. Hence, no experimentation was required by Strauer regarding the delivery technique.

The PTO opines at pages 11-13, ¶15 of the Office Action that, "...it is still clear that considerable experimentation was done, if not by Strauer, then by others, in order to determine the effective cell population" without citing any authority. Appellant teaches that the entire array of bone marrow mononuclear cell components contribute to the regeneration of ischemic tissue; and such teaching is consistent with Orlic, Strauer, and Dohmann, which confirm that mononuclear bone marrow cell components promote new cardiac muscle and new artery growth. In the absence of evidence provided by the PTO showing experimentation "by others," the record supports Appellant's position that no undue experimentation would be required in order for one skilled in the art to practice the method defined in the claims on appeal. Rather, one skilled in the art having read Appellant's specification would readily understand that the placement of the entire array of stem cells harvested from bone marrow or blood in the body of a human patient would cause the formation of a new artery. Of course, such placement techniques are well known and documented in the art, leaving no need for more than routine experimentation. In this regard, see the comments contained in the article in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Progenitor Cell Transplantation and Function following Myocardial Infarction" (of record and attached hereto as Exhibit B). Exhibit B is a complete copy of the above article and is attached because the copy furnished by the PTO is incomplete.

In the above-mentioned article, Mr. Pollman indicated that the TOPCARE study, "...uses a simple syringe injection system loaded with...bone marrow..." [Emphasis added]. Appellant believes that such quotation supports its position that no more than

routine experimentation would be required to administer the materials of the claimed invention.

Contrary to the PTO's assertion at page 11, first line of ¶15 of the Office Action, Strauer—just like Appellant—does not disclose that stem cell population is critical and does not describe any experimental protocol for selecting and isolating certain cells from the entire cell population described for the treatment therapy.

The concept of containment to prevent backflow and prolong contact time is clearly taught by Nabel. Thus, contrary to the PTO's assertion, it is clear that Strauer did not require or perform any experimentation to choose an appropriate delivery system or devise a containment system that would prevent backflow of cells and thus provide a prolonged time for cell implantation. Rather, such choice constitutes no more than the routine use of a well-established delivery system.

Strauer does not describe using any experimental protocol to determine appropriate cell population, i.e., there is no requirement for using a specific subset of bone marrow stem cells. Regarding time of treatment, Strauer does not disclose that determining time of treatment required experimentation. It is clear from the record that the treatment of myocardial infarction (MI) in human patients exhibiting either acute or chronic disease is considered. Strauer elected to treat patients from five to nine days after suffering an MI. Note that in a later publication in Circulation of Strauer et al. in 2005 (hereinafter "Strauer 2005" and of record) discloses treating chronic MI in patients that had transmural MI some 27 months earlier. Again, no experimentation regarding treatment time was noted. It is evident that the time of treatment following an MI is not a critical variable, and undue experimentation would not be required. To the extent that the

PTO may be relying on Strauer to establish that the time of administration is critical, Appellant points out that Strauer 2005 is the “best evidence” in regard to whether time of treatment in human patients is critical. Strauer 2005 teaches that stem cells can be used to successfully treat MI in human patients suffering either acute or chronic disease. Moreover, Isner ’887 also does not indicate that time is critical in the treatment of humans exhibiting ischemic heart tissue, and this was not viewed as an impediment by the PTO. Thus, the PTO’s conclusion that “great quantities of experimentation” would be required to practice the claimed invention is not supported on the record and is fatally flawed.

The PTO’s reference at pages 11-13, ¶15 of the Office Action to “dark data,” while interesting in a conspiratorial sense, is entitled to zero evidentiary value. Clearly, it defies logic to utilize a negative to support a positive. The PTO’s statements relating to asserted widespread “unethical” practices of researchers in the medical arts is truly amazing and perhaps revealing of a deeper social problem. Does the PTO actually take the position that medical professionals, like Dr. Strauer and his colleagues, commonly withhold “failed” data that could be helpful/harmful to others? Equally amazing is the present PTO Examiner’s self imposition of Official Notice. Apparently, the present PTO Examiner is not familiar with the legal concepts of judicial notice (or administrative notice in the context of patent prosecution) and burden of proof. If an examiner wishes to establish himself/herself as an expert based upon his or her personal experience as a former researcher regarding the practice of “unethical” data reporting, then be advised that the proper evidentiary vehicle is in the form of a sworn instrument, along with its legal implications. Unethical practices such as these typically become “bread and butter”

for product liability lawyers. In the absence of such a sworn statement, the present PTO Examiner's taking administrative notice does not rise to the level of evidence, should be disregarded by the Board; and consequently, be treated as nothing more than unsupported hubris.

The PTO posits in the Office Action at pages 13 and 14, ¶¶16-17 that the specification does not provide "...guidance for, or even suggest the use of bone marrow stem cells, any kind of stem cells, or cells of any kind, to grow an artery or repair a heart." Attention is directed to pages 47 and 48 of the specification wherein guidance is provided for forming soft tissue organs by direct differentiation and morphogenesis by reimplanting a patient's own cells, such as "growth of an artery" (page 48, line 3) which in "some cases [comprise] stem cells" (page 48, line 13). It is illogical for the PTO to contend that pages 47 and 48, particularly when read in light of the specification as a whole, provide no guidance to one skilled in the art for reimplanting a patient's own stem cells and growing an organ, such as an artery, by direct differentiation and morphogenesis. Guidance for harvesting stem cells from the bone marrow of the patient for reimplantation to promote morphogenesis of soft tissue is provided on pages 40-42 of the specification. One skilled in the art reading the instant specification's teaching of using stem cells harvested from the bone marrow or blood of the patient would understand that the claimed invention distinguishes from Isner '887 by describing using unfractionated (global) bone marrow mononuclear cells. As pointed out earlier, there is no basis in fact for the PTO to determine that the instant specification provides guidance to one skilled in the art for implanting anything other than the entire array of bone marrow derived cells harvested from the patient's bone marrow. Whether one uses the

terms “global,” “whole population,” “unfiltered,” and “unfractionated” matters not a whit. Certainly, unlike Isner ’887 and Asahara, the concept of isolating/separating of a component of the entire array of bone marrow stem cells is not implicitly or explicitly contemplated or described in the instant specification. Reading and interpreting the disclosure to include such concept is improper because it distorts the reasonable/intended guidance provided to one skilled in the art by Appellant’s specification. Isner ’887 serves as contemporary prior art for apprising one skilled in the art of regenerative medical procedures for direct intramuscular injection of DNA encoding VEGF or EC progenitor stem cells to promote angiogenesis by increasing capillary blood vessels in ischemic tissue. One skilled in the art being so apprised and reading the instant specification would understand that Appellant has provided sufficient information, i.e., the process steps, instrumentation, and stem cell compositions essential to grow an artery to enable one skilled in the art to practice the method set forth in the claims in issue.

The PTO’s arguments bridging pages 14 and 15, ¶¶17-19 of the Office Action attempting to establish that “the scientific considerations of handling, dosage, carriers, etc. are completely different” for handling nucleic acids *vis-à-vis* cells are inapt at best. The Examiner’s “apples to oranges” analogy misses the point and misstates Appellant’s position. The point that Appellant was making was that while the selection of materials is different, the administration and apparatus is substantially similar, if not identical, for genes and cells. A simple hypodermic syringe is used in each instance. The PTO has not provided any evidence to rebut this point. Those skilled in the art are well versed in the compositional differences, handling, and administering techniques involved in employing these therapeutic agents. The PTO’s argument presupposes that the disclosure of Isner

'887 is limited solely to the claims therein – that the knowledge gleaned by one skilled in the art reading Isner '887 would be so limited. This is clearly not the case. Isner '887 at column 2, line 9 to column 3, line 27 clearly teaches that it was known that intramuscular injection of DNA encoding VEGF into ischemic tissue induces the growth of capillary blood vessels and describes as an improvement to the state of the art an *alternative* method for promoting angiogenesis involving *in vivo* implantation of cells, specifically EC progenitor stem cell fractions into sites of blood vessel injury. Isner '887 describes obtaining bone marrow–derived mononuclear cells containing stem cells by using techniques similar to those previously used in the medical arts for obtaining hematopoietic stem cell (HSCs) therapeutic compositions commonly used in bone marrow transplantation. These are the same common techniques used by Strauer and contemplated in the instant specification for recovering BMC's. Isner '887 differs from such common practice by separating EC progenitor cells for implantation into patients. Isner '887 teaches that once implanted, the EC progenitor cells selectively migrate to the foci of blood vessel injury without any further manipulation by the medical practitioner. Notwithstanding PTO classification, one skilled in the art would understand from reading Isner '887 and the instant specification that stem cell therapy and gene therapy are considered alternative methods for promoting angiogenesis by those skilled in the medical arts.

The PTO's arguments raised at pages 16-19, ¶¶20-23 of the Office Action are moot in regard to Appellant's elected invention cells and more specifically stem cells presently on appeal. Pursuant to the PTO's prior restriction requirement, Appellant elected "cells" as the growth factor for examination purposes. Such election resulted in

claims 240-242, directed to the growth factor “genes,” being withdrawn from consideration by the PTO. Attention is also directed to the fact that numerous restriction requirements between “gene” and “cell” growth factors have been consistently made by the PTO. In fact, continuation application Serial No. 11/605,153 filed November 28, 2006, contains such a restriction requirement and thus confirms that the PTO considered “cells” to be a specie of growth factors. Accordingly, the present PTO Examiner’s allegation that cells are somehow not growth factors is manifestly inconsistent with prior PTO determinations, including in this application and its continuation application, as well as the plain meaning of such term set forth in Appellant’s specification. Once the present PTO Examiner reads the specification in a reasonable manner, there can be no doubt that cells are a growth factor. See Phillips v. AWH, Corp., supra. In any event, while dictionary definitions of the term “growth factor” maybe of academic interest, they do not alter the status of the elected invention. The error arose from the PTO’s continuing rejection of unelected generic growth factor claims. Appellant responded to the rejection of the generic claims to quiet any possible estoppel issues.

As pointed out earlier, the term “growth factor,” as used by Appellant in the context of the described and claimed invention, is defined on page 20 of the instant specification as comprising a composition which promotes the growth of soft tissue, and specifically, as an angiogenesis promoter for artery growth. Appellant’s disclosure is not inconsistent with that of Alberts et al. in Molecular Biology of the Cell, 4th Ed., 2002, (of record). Moreover, Dr. Isner, as well as Declarants Drs. Richard Heuser and Andrew E. Lorincz, recognized that both cells and genes promote soft tissue growth. Both DNA encoding VEGF and EC progenitor cells are described by Isner ’887 as promoting the

growth of soft tissue, capillary blood vessels. Clearly, one skilled in the art apprised of the teachings of Isner '887 relating to the properties of both genes and cells when reading the instant specification would find ample guidance for injecting either DNA encoding VEGF or cells, such as stem cells, for promoting the growth of arteries to treat ischemic tissues in humans. This conclusion comports with Paragraphs 7 of Declarants' opinions (Fourth Supplemental Declaration of Dr. Heuser and Third Supplemental Declaration of Dr. Lorincz, both of record). These declarations express the opinions that one skilled in the art would understand that Appellant's specification disclosed the concept that intramuscular injection would be applicable for use in growing an artery in a human patient regardless of whether the composition was a "gene; cell, including stem cells such as bone marrow stem cells."

The PTO's contention that, "The specification provides no guidance along the lines of the details worked out by Strauer" is misplaced. Firstly, none of the claims on appeal require the use of an angioplasty balloon catheter; and, therefore, it is improper for the PTO to look solely to Strauer for guidance. A more proper model to compare with would be Isner '887. Secondly, application Examples 18, 19, and 36 describe a detailed regimen for treating a patient with a damaged heart by injecting a nucleic acid growth factor for promoting artery growth, which includes mode, dosage, and means for evaluating success of treatment, which is similar to the regimen disclosed by Isner '887. Specifically, Example 18 (page 53, line 25 to page 54, line 4) describes a regimen wherein cDNA is injected slowly and which employs a containment system to prolong contact time and to avoid leakage or wash away. The application disclosure also teaches on pages 40-42, 47, and 48 utilizing autologous stem cells harvested from bone marrow

and blood of the patient (self-cell therapy) or from cell cultures (allogenic) to grow organs, i.e., arteries, by differentiation and morphogenesis (page 48).

The Office Action, at pages 19-25, ¶¶ 24-33, addresses the calculus employed by Appellant relating to guidance provided by the specification for determining dosages of cells (stem cells) for promoting morphogenesis.

At the outset, it is axiomatic that claims do not have to recite dosage levels where dosage levels would be understood by those skilled in the art. It is clear from Strauer, as well as prior art medical practices relating to bone marrow transplants in general, that it is difficult to over-dose, especially when dealing with implantation of autologous BMC's. As succinctly stated in MPEP Section 2164.01(c):

It is not necessary to specify the dosage or method of use if it is known to one skilled in the art that such information could be obtained without undue experimentation. If one skilled in the art, based on knowledge of compounds having similar physiological or biological activity, would be able to discern an appropriate dosage or method of use without undue experimentation, this would be sufficient to satisfy 35 U.S.C. 112, first paragraph.

The PTO, at page 19, ¶24 of the Office Action, challenges Appellant's reliance upon Examples 18, 19, and 36 for guidance in selecting cell dosages. Appellant believes that these Examples provide guidance of dosages and methods of use for compositions in general described in the specification having the requisite physiological activity for inducing angiogenesis. Moreover, one skilled in the art would understand from Isner '887 that intramuscular injection of DNA encoding VEGF or EC progenitor cells (stem cell fractions) promote capillary blood vessel growth. Examples 18, 19, and 36 of the specification describe methods for carrying out the invention including dosage amounts

for compositions used to promote artery growth and heart repair. Appellant's specification describes new artery growth and heart repair by direct intramuscular injection of DNA encoding VEGF into ischemic tissue in dosage ranging from approximately 250 micrograms (Examples 18 and 36) to approximately 500 micrograms (Example 19). This weight readily converts to a dosage of cells ranging from approximately 6.25×10^6 and 12.5×10^6 . Available off-the-shelf cDNA clones (nucleic acids) are directly injected into either the cardiac muscle (Example 19) or the coronary artery (Example 36). Each example describes repairing a damaged heart by forming a new artery which results in increased coronary blood flow. Each example also discloses slowly injecting the growth factor to avoid any carry away. Example 18 discloses that a containment system may be used. While these examples employ nucleic acids, one skilled in the art reading the specification and being apprised of Isner '887 and Asahara, which teach that cells, i.e., EC progenitor stem cells, possess similar/equivalent physiological activity to genes (nucleic acids), in forming a new artery and repairing a dead or damaged portion of a heart, would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. Note in this regard that Strauer discloses injecting six (6) to seven (7) times with 1.5 to 4×10^6 cells without disclosing any difference in results over the entire dosage range. Therefore, there is no significant clinical difference between Appellant's 6.25 to 12.5×10^6 and Strauer's 9 to 28×10^6 dosage ranges. Further, such skilled person would understand that intravenous or intraluminal administration routes would generally require larger doses than the direct injection route of Examples 18, 19, and 36, and, for example, simply doubling the dosage to 12.5 to 25×10^6 cells would essentially encompass Strauer's

entire range.¹ It is clear from Strauer that there is no risk for over-dosing, particularly when using autologous BMC's, which are contemplated in Appellant's specification. The nontoxicity of autologous BMC's dosages was established over decades in the medical arts in the treatment of cancer. Further evidence of the reasonableness of the calculus used by Appellant for extrapolating dosages across the range of cells and genes can be found in Isner '887. In this regard, note that the cell and nucleic acid doses disclosed by Isner '887 fairly compute on a weight to weight basis. For example, using 2,000 micrograms as a preferred dosage of nucleic acid described by Isner '887 one skilled in the art applying Appellant's calculus could extrapolate to a cell dosage of about 50×10^6 , which falls within the range specified for cells by Isner '887². cf. In re Bundy, 642 F. 2d 430, 434, 209 USPQ 48, 51-52 (CCPA 1981).

Appellant believes that the present PTO Examiner's opinion proffered at pages 21-23, ¶¶28-30 of the Office Action in regard to the dosage extrapolation is unwarranted and lacks proper decorum. The Third Supplemental Declaration of Richard Heuser (of record and originally filed in co-pending application Serial No. 10/179,589) and the Second Supplemental Declaration of Andrew E. Lorincz (of record and originally filed in co-pending application Serial No. 10/179,589) confirm and establish as a fact that the extrapolation was long known in the art and provides an expert opinion that Appellant's

¹ The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 19 and 36 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6 , respectively.

² The conversion for Isner'887 dosages of nucleic acids to corresponding dosages of cells was conducted as above. The 2,000 microgram dosage was converted to pg by multiplying by 10 equals 2000×10 pg. An average cell weight of 40pg was used for nucleic acid as consistent with the above. The conversion was

reliance thereon is reasonable. Notwithstanding the clairvoyance of the present PTO Examiner expressed at page 23, ¶30 of the Office Action, the above-mentioned respective Declarations of Drs. Heuser and Lorincz speak for themselves and confirm the reasonableness of Appellant's conversions. It matters not a whit whether or not Drs. Heuser's and Lorincz's specialties reside in expertise in molecular biology. The declarations are what they are—declarations expressing the opinions of experts in the medical field—not of a microbiologist having no experience in the medical field and consequently having no practical experience and knowledge of dosage practice in the medical art. It is again emphasized that the extrapolated dosages compare favorably (overlap) with the dosages of global bone marrow cells used by Strauer for promoting angiogenesis in treating myocardial infarction in human patients and the cell dosages used by Isner '887 for promoting endothelial tissue growth, thereby confirming the reasonableness of the respective Declarants' opinions.

In summary, the PTO's *ad hominem* criticism of Appellant's conversion fails to adequately give weight to its evidentiary value. Appellant's evidence establishes as a material fact that physicians have long used conversion charts/formulas for estimating dosages of cells from nucleic acids and vice versa. It is clear from the record that cell survival and differentiation are not paramount considerations in determining cell dosages because the general practice is to employ multiple dosages because stem cell overdosing has not proved to be problematic. Most importantly, there is no guidance proffered in Isner '887 regarding the need to employ disparate treatments for the delivery of cells *vis-*

then made by dividing 2000×10^6 by 40 to arrive at an equivalent cell dosage of 50×10^6 , which dosage falls within the range specified for cells by Isner'887.

à-vis genes. Those skilled in the art are aware that safe dose ranges have been established over years of medical practice directed to bone marrow transplant of cells.

On page 26, ¶35 of the Office Action, the PTO states that neither the specification's use of prophetic examples nor claiming something that has not been done before are the basis for the enablement rejection. It has been Appellant's understanding from the beginning that prophetic disclosures are permitted under the rules, statute and case law. However, the PTO concludes, without further explanation, that the lack of actual examples "contribute significantly," i.e. was a contributing factor along with "other Wands factors" in determining of lack of enablement. It is the burden of the PTO to specifically and precisely point out why the absence of specific working examples, along with any "other Wands factors" support a *prima facie* case of non-enablement. Appellant submits that the PTO has not met such burden.

While Appellant agrees with the PTO that the medical arts in general and the physiological reactions involved may be complex, the practice of the claimed invention is straightforward. In the previously-mentioned publication entitled, "Progenitor Cell Transplantation and Function following Myocardial Infarction," Dr. Richard Heuser, a Declarant of record, in connection with the TOPCARE study stated that, "The first time I saw this technique presented by the group in Frankfurt, I was astonished at how simple it actually was." Such quotation, although contained in the full copy of the above article, was omitted in the copy furnished by the present PTO Examiner. In any event, the called-for cells, e.g. bone marrow cells, the methods for administering, and the particular apparatus required for administering the cells are old and commonly used in cell therapy; and thus, the practice of the invention is not deemed to be complex.

At page 26, ¶36 the Office Action, the PTO acknowledges that it, “cannot, and does not, demand human clinical trials to demonstrate enablement...” Appellant appreciates such pronouncement since the previous actions by the PTO lead Appellant to believe that the method and manner of making the claimed invention was the predominant contributing factor in the PTO’s determination of lack of enablement. Appellant referred to the claims in Kornowski et al. U.S. Patent No. 7,097,832 (hereinafter “Kornowski” and of record) as being drawn to treating humans as prophetic, not to challenge the enablement of such patent. It is and has been Appellant’s position that actual working examples, whether animal or human, are not required.

At page 27, ¶37 of the Office Action, the PTO puts forth the proposition that there is a higher “enablement” standard required by the statute for “cases that involve unpredictable factors such as most chemical reactions and physiological activity” while citing case law presumably “codifying” such a higher standard. In other words, the PTO is placing a higher burden on Appellant to support enablement because of the nature of the claimed invention. The PTO is relying on case law because the first paragraph of §112 does not embody such a separate requirement for chemical and physiological related inventions *vis-à-vis* other classes of inventions. What is certain is that the question of enablement must be determined on a case-by-case basis taking into consideration the facts presented. The specification discloses all the information that is needed for one skilled in the art, for example, to: 1) select bone marrow stem cells harvested from the patient; and 2) intramuscularly inject said stem cells into sites of ischemic tissue for promoting differentiation and morphogenesis into new blood vessels, i.e., arteries, and to grow cardiac muscle.

The import of the references cited by the PTO at page 28, ¶38 of the Office Action as evidence of the uncertainty in the art as to the “precise population of cells that give rise to endothelial cells” is not understood. The population of cells included under bone marrow stem cells was discussed above on page 25 of the instant Brief in connection with pages 13 and 14, ¶¶16-17 of the Office Action. Such population was thoroughly discussed and distinguished over the sole CD 34+ progenitor cells of Isner ‘887 and Asahara. In regard to Isner ‘887 and Kornowski, only the latter relies upon the entire array of bone marrow cells as claimed in the present application. Although Kornowski uses the same population of bone marrow cells as claimed, Kornowski is not a competent reference due to its filing date. Isner ‘887, as discussed earlier, utilizes a different composition consisting of EC progenitor cells. The PTO failed to articulate how such references are relevant to establishing a *prima facie* case of lack of enablement of the subject matter called for by the appealed claims *vis-à-vis* the objective enablement provided by the instant specification. To the extent the PTO is challenging the predictability of Appellant’s described heart repair by promoting artery growth through implanting BMC’s, Dohmann describes the results of implanting BMC’s to provide heart repair and thus suffices to allay such challenge. Dohmann provides autopsy proof that such heart repair involves artery growth. Regarding “prior art,” none has been identified or cited by the PTO against Appellant’s claims—and for good reason. Appellant was the first to disclose and claim a method for human heart repair by implanting cells, such as stem cells, and growing a new artery. The PTO has also cited and relied upon the post-filing Rabelink et al. publication entitled, “Endothelial Progenitor Cells: More Than an Inflammatory Response?” in Arteriosclerosis, Thrombosis, and Vascular Biology,

(hereinafter “Rabelink” and of record) to support a lack of enablement conclusion. The PTO alleged that this publication constitutes subsequent experimentation that evidences a factual uncertainty as to which cells give rise to endothelial cells. A review of Rabelink evinces that this publication is directed to the effect of circulating endothelial cells in response to hypoxia and the possibility of cardiovascular risk factors when therapeutically mobilizing such cells. There is nothing in Isner ’887 or Rabelink that teaches that EC progenitor cells will result in artery formation. Dohmann revealed no adverse histological findings after an eleven month follow-up of human patients treated with bone marrow stem cell implantation. Moreover, Dohmann constitutes the best evidence because, unlike Isner ’887 and Rabelink, Dohmann’s process, materials, and results correspond to the claimed invention. Any uncertainty is clearly put to rest by the autopsy findings disclosed by Dohmann. The PTO’s summary determination that undue experimentation would be required to provide the art skilled with “factual certainty” that the inventor, Dr. James P. Elia, was in possession of the “complete invention as it will be used in practice” lacks factual support. The PTO has failed to critically review the entire record and present a factual analysis which supports such determination.

The PTO in the Office Action at page 29, ¶39 foists the contention that the specification fails to disclose with specificity “which cells would work” for promoting growth of an artery by gratuitously disparaging unclaimed inventions described by the inventor. Appellant has continuously argued, and cited legal authority supporting the proposition, that the entire specification disclosure must be considered by the PTO when determining whether the claimed subject matter on appeal reasonably finds descriptive and enabling support therein. However, such argument does not open the door to the

present PTO Examiner's gratuitous and sometimes derogatory expressions of opinion concerning unclaimed inventions. The PTO examination process is not and should not be an adversarial proceeding.

The PTO's contention that the specification provides no guidance for selecting cells that would or would not work when practicing the invention simply ignores the written text of the disclosure. The specification clearly teaches that pluripotent stem cells, such as bone marrow mononuclear cells, promote the growth of arteries and cardiac muscle. Workers as early as the Caplan's 1991 publication in Journal of Orthopaedic Research, entitled, "Mesenchymal Stem Cells" (of record) reported the pluripotency characteristic associated with bone marrow stem cell populations, i.e., their ability to form multiple soft tissue types. The pluripotent characteristics of hematopoietic stem cells harvested from bone marrow were recognized and have been utilized in the treatment of cancer for decades. One skilled in the art reading the specification would understand that pluripotent stem cells are essential for forming the multiple tissue types that are required for organ formation. Consequently, a person skilled in the art would understand that unipotent stem cells, such as the EC progenitor cells employed by Isner '887 and Rabelink, would not differentiate into an organ, such as an artery, because an artery contains muscle tissue. Thus, the PTO's erroneous focus upon Isner '887 and Rabelink is not relevant to the claimed invention, which requires formation of an artery and cardiac muscle.

The PTO criticized Appellant's reliance upon Examples 11 and 14-16. Appellant referred to Examples 11 and 14-16 as disclosing the use of bone marrow stem cells for promoting differentiation and morphogenesis into tissues and organs. Such Examples

were not relied upon to specifically show artery growth, although those skilled in the medical art would understand that growth of a tooth, kidney, or eye requires the growth of an artery. This further comports with the disclosure in the specification at pages 47 and 48 and paragraphs 7 of the Fourth supplemental Declaration of Dr. Heuser and the Third Supplemental Declaration of Dr. Lorincz (see page 29 of the instant Brief) which directs the use of autologous stem cells.

At page 30, ¶40 of the Office Action, the PTO refers to page 37 of the specification as failing to suggest using cells to grow an artery. Appellant has not asserted that guidance is provided by such text for using a cell to grow an artery. Lost is the fact that line 26 on this page teaches that growth factors include cellular products and their derivatives. Appellant's general statement that "any host cell, cloned cell, cultured cell, or cell would work," was not intended to support the notion "that any cell can do anything." While such disclosure is prophetic in nature, it has at least found support in recent cell therapy studies involving fat cells and induced pluripotent cells such as skin cells.

At pages 30 and 31, ¶¶41 and 42 of the Office Action, text from the specification is correctly cited. The Office Action in ¶42 mischaracterizes the intended meaning of this disclosure by stating that it only applies to the use of "skin cells." To one skilled in the medical arts, Appellant's disclosure teaches that a patient's own cells can be used to induce growth of an organ or function-specific tissue and that germinal cells and stem cells are contemplated to be used for inducing direct differentiation and morphogenesis into an organ. One skilled in the art would readily understand that reimplanting a patient's own stem cells comprises implanting autologous bone marrow stem cells.

While the Examiner may not understand what the term “germinal cell” includes, those skilled in the art are aware that a germinal cell is a cell which divides into other cells. In plain terms, a germinal cell is a cell that is capable of differentiating. Thus, the language “germinal cells (and in some cases, stem cells)” clearly defines cells that are capable of direct differentiation and morphogenesis into an organ, e.g., pluripotent cells capable of inducing growth of multiple tissues. The PTO’s position in ¶43 of the Office Action that even if the disclosure can be said to disclose using stem cells to grow an artery, it merely suggests an idea without teaching the skilled artisan how to do it lacks merit. Once the concept of using bone marrow stem cells to grow arteries is revealed to a skilled artisan, the mode and mechanisms for implantation are readily within the skill of such a person. The PTO in the Office Action in ¶44, appears to concede that it is a fact that cells are reasonably “living organisms.” Therefore, the specification on page 20 reasonably defines “growth factor” as including cells that promote organ growth, which comports with Alberts’ definition of growth factor.

At page 33, ¶45 of the Office Action, the PTO necessarily raises an issue that actual experiments (working examples/clinical trials) are required establish enablement for the claimed invention yet fails to specifically identify what protocol is missing from Appellant’s specification that would prevent one skilled in the art from practicing the claimed subject matter. The PTO’s remarks involving “writing it down,” absent evidence or sound reasoning, is insufficient to overcome the objective enablement provided by the specification. In re Marzocchi, 58 CCPA 1069, 439 F.2d 220, 169 USPQ 367, 369-370 (1971). Apparently, the PTO fails to appreciate that the act of “writing down” a “prophetic” example, which describes an embodiment based upon predicted results rather

than work actually conducted, is sufficient to satisfy a constructive reduction to practice. See MPEP 2164.02 and cited case law that stands for the proposition “the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it.” In re Chilowsky, 29 F. 2d 457, 461, 108 USPQ 321, 325 (CCPA 1956). Nor does the use of prophetic examples automatically render a specification non-enabled. The burden is on the PTO when challenging enablement to show by clear and convincing evidence that the prophetic examples, when combined with the disclosure as a whole and in view of the knowledge in the art as of the filing date of the application, does not meet the enablement requirements of the statute. It is clear on this record that the PTO has not discharged such a burden. Cf. Amgen, Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200, 18 USPQ 2d 1016 (Fed. Cir.), cert. denied, 502 U.S. 856, (1991). The PTO has acknowledged that it determines enablement on a case-by-case basis and does not require even *in vivo* evidence. This is clear from Kornowski, which contains claims drawn to a cell therapy treatment of humans requiring the implantation of bone marrow stem cells in the heart to grow collateral blood vessels based on a prophetic disclosure. Not being skilled in the medical art, the present PTO Examiner appears to be challenged by the term “cascade of genetic material” as used in the specification at page 37. The Board’s attention is again directed to the quotation of William O’Neil set forth on page 19 of the instant Brief, which is evidence that those skilled in the medical art reading the specification would fully understand the terminology used by Dr. Elia in describing the necessary *in vivo* cascade of processes that allow implanted cells to regenerate in a patient’s body. Also as

mentioned above, in connection with William O'Neil's quotation, the present PTO Examiner omitted such quotation in the copy of the article furnished to Appellant.

The PTO Examiner at pages 34-37, ¶¶47, 48 of the Office Action cited two internet articles published in The Journal of Invasive Cardiology, Vol. 17, July 1, 2005, entitled, "Tissue Engineering and Interventional Cardiology" and "Progenitor Cell Transplantation and Function following Myocardial Infarction." In the Office Action, the Examiner furnished Appellant with copies of the two articles (attached hereto as Exhibits A1 and B1, respectively). Such copies contain less than the complete content of the published articles and lack proper context. Appellant has provided complete copies of the published articles in attached Exhibits A and B, respectively, for the Board's consideration and comparison to the version furnished by the present PTO Examiner.

The two above-identified articles (Exhibits A1 and B1) are relied on by the PTO to challenge Appellant's assertion of post-filing success for the claimed method. The PTO contends the furnished excerpts show that some seven years after the filing date of the instant application, skilled workers in the art voiced concerns about cell choice, dosages, time of treatment, implantation apparatus and cell survival were unanswered. Appellant's comparison of the full text of the articles with that of the versions furnished by the PTO clearly evinces that the present PTO Examiner has artfully selected portions of the context, while omitting other portions thereof, in an apparent attempt to spin the meaning of the text. Such editing raises a serious question in regard to the probative value of the furnished material, as well as the administrative process of the PTO.

Appellant has reviewed the limited context from the excerpts presented at pages 34-37 of the Office Action but disagrees that the verbage thereof rises to the level of

evidence supporting non-enablement. Most of the comments concerned the BOOST, TOPCARE and Bio Heart trials. The latter body of work is dissimilar from the present invention in that it used a skeletal muscle myoblast product. Matt Pollman from Guidant Corp. described the BOOST method, “as a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries.” Pollman does not indicate that any further manipulation was necessary. Appellant has consistently taken the position that the Strauer publication relied upon by the PTO describes little if any experimentation required to practice the disclosed implantation of bone marrow stem cells. Appellant makes the following comments regarding the excerpts presented by the PTO.

- The first quoted statement of Mr. O’Neil is merely asking a question that had been previously answered by Strauer.
- As O’Neil’s second quoted question, neither Andreas Zelher (Guidant, Frankfort) nor Strauer reported any problem with cell hypoxia.
- Mr. O’Neil’s third question virtually confirms Appellant’s argument that the specification teaches using unfiltered bone marrow.
- Mr. Nikol’s comments sound like professional envy rather than critical analysis of bone marrow implantation.
- Mr. Gonschior’s comments merely affirm that intravenous infusion would be the simplest method while Strauer’s endocardial delivery may be the most efficient. These comments mirror the views expressed by Strauer.

- The quoted comments by Holmes merely express his criticism of premature human trials and appears to be especially directed to systemic infusion of cells.
- Mr. Whitlow's quoted comments are purely theoretical and do not evince that his opinions are based on the performance of any experimental or clinical trials. The autopsy findings described in Dohmann show that Whitlow's theoretical premises are not well founded.

It is puzzling that the PTO can conclude from such selective utterances that "[t]here was a general agreement that more experimentation was needed." This is particularly telling when one understands that the later work of Dohmann and Kornowski closely parallel Strauer's work.

One final point remains. What is most disturbing to Appellant regarding the PTO's use of these two articles is the omission of information favoring enablement of Appellant's claimed method. For example, the PTO omitted the statement by Nikol that, "cells are considered a blood product" and the statement by O'Neil that, "...because these bone marrow cells are pluripotential..." A further example is the spontaneous utterance of Dr. Heuser that "[t]he first time I saw this technique presented by the group [TOPCARE] in Frankfort, I was astonished at how simple it actually was," and Pollman's statement that, "a simple syringe injection system" was used for implantation. It is tempting to speculate that the present PTO Examiner's omission of such comments by Mr. Pollman and Dr. Heuser could be attributed to the Examiner's carefully refraining from providing evidence backing up arguments made by Appellant in the record that once one skilled in the art realizes that bone marrow promotes the growth of arteries the

delivery of the bone marrow is simple. In any event, the above utterances indicate that the treatment is not complex as alleged by the PTO. The answer to the PTO's irrelevant question, "Why didn't "[Dr. Heuser] enlighten his colleagues?" is straightforward. Being a patentee in his own right, Dr. Heuser fully comprehends his duty in regard to confidential information, even if the Examiner is dismissive of such duty. Matt Pollman, an employee of Guidant, was aware of confidentiality obligations regarding privileged information, as were all of the others. See the Pullman comment near the bottom of the first page of the "Progenitor Cell Transplantation and Function following Myocardial Infarction" article (Exhibit A). In addition, an opinion regarding enablement based upon the disclosure of a patent application is distinct from optimizing medical processes and continuing research involving such processes. The Examiner's query misses this point.

The PTO asserts, at page 38, ¶49 of the Office Action, that at the time of filing the instant application, "the notion that the new result, cardiac muscle, and artery growth, can be achieved using old materials (bone marrow) and old methods (injection)" was nothing more than "a germ of an idea." The Examiner, relying on Genentech Inc. v. Novo Nordisk A/S, (CAFC) 42 USPQ 2d 1001 (1997), asserted that, "The courts have also stated that "[t]ossing out the mere germ of an idea does not constitute an enabling disclosure...[R]easonable detail must be provided in order to enable members of the public to understand and carry out the invention." If the specification had done no more than to generally suggest that the use of some unidentified composition could grow soft tissue, such cardiac muscle and an artery in a human patient, such general suggestion would constitute tossing out a germ of an idea. The above unsupported conclusion of the

PTO overlooks the fact that Dr. Elia disclosed an inventive concept of implanting cells, including bone marrow stem cells, into an infarcted heart of a human patient to grow cardiac muscle and an artery. The specification clearly discloses the placement of specific cells, such as bone marrow stem cells, specific delivery systems, such as injection through a hypodermic syringe, and specific placement sites, such as adjacent to a dead or damaged portion of a heart. A reasonable skilled person reading the specification would understand that it describes more than tossing out a mere germ of an idea. The Declarations of Drs. Heuser and Lorincz comport with Appellant's position in this regard. Hence, the PTO has committed error by merely quoting a legal conclusion contained in a judicial decision without providing the necessary factual basis and evidence to support such legal conclusion.

Rejection of Claims 288-290
Under 35 U.S.C. §112, first paragraph

Claims 288-290 stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. Appellant disagrees with such rejection. Appellant hereby repeats and relies upon the above presented remarks regarding the rejection of claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, and 280-285. Additionally, Appellant submits the following remarks in support of the enablement of claims 288-290.

Claims 288, 289, and 290 differ from the claims from which they depend upon, namely claims 261, 268, and 269, respectively, by requiring autologous cells placed into the heart of the patient by injection (claim 288), by requiring autologous cells with

injection said cells at a site adjacent a dead portion of a heart (claim 289), and adjacent to a damaged portion (Claim 290). These claims are drawn to the narrowest embodiment of the invention by requiring specific cells, specific sites, and specific modes of administration. The disclosure embracing the subject matter of these claims can be found, for example, in the specification at pages 21, line 4-15; page 32, lines 9-11; page 33, line 8-10; page 40, line 27 to page 42, line 27; page 44, lines 12 and 13; page 46, lines 3-10; page 47, line 22 to page 48, line 15; Example 19 on page 55, line 14 to page 65, line 25; and Example 36 on page 62. One skilled in the art would be fully able to make and use the invention having the scope of claims 288-290.

Appellant submits that, on this record, the PTO has failed to provide sufficient evidence for supporting a *prima facie* case of lack of enablement and the rejection for lack of enablement should be reversed.

Assuming, *arguendo*, that the PTO somehow met the burden of establishing a *prima facie* case of lack of enablement, Appellant believes that any such case has been rebutted by the weight of the evidence contained in the nine Declarations of two experts in the field, Dr. Richard Heuser and Dr. Andrew E. Lorincz (all of record). Such Declarations are set forth in the Evidence Appendix (Items 13-14 and 20-26). The conclusions set forth in the respective Declarations establish an objective fact that is highly material to a determination of enablement. These two highly skilled medical experts read relevant portions of the specification, including generic as well as those drawn to elected and non-elected species, and reached the determination that one skilled in the medical arts, armed with the knowledge in the disclosures, would be enabled to practice the claimed method and to predictably anticipate the results defined therein

without need for resorting to undue experimentation. See paragraphs 5-7 of the Third Supplemental Declaration of Dr. Lorincz and paragraphs 5-7 of the Fourth Supplemental Declaration of Dr. Heuser.

The PTO, at page 34, ¶46, states that the declarations of Dr. Heuser and Dr. Lorincz are accorded no weight. The PTO contends that opinions of experts in regard to the ultimate legal conclusion of enablement are entitled to no weight, citing In re Lindell and In re Chilowsky for precedent. The PTO refers to pages 38-42 of the Examiner's Answer dated January 24, 2008, for detailed reasoning. Case law was cited as standing for the proposition that enablement is a question of law. However, it is clear from MPEP 2164.05 that declarations are evidence that must be considered and that weight must be accorded based on the factual evidence presented therein supporting a conclusion of enablement. The Court in In re Buchner, supra, held that "expert's opinion on the ultimate legal conclusion must be supported by something more than a conclusory statement." In In re Buchner, the PTO determined that the specification lacked enablement because elements necessary for carrying out the invention were neither disclosed therein nor well-known to those of ordinary skill in the art. The Court, while recognizing that the Buchner specification need not disclose what is well known in the art, agreed with the PTO that unless the identified missing elements were well-known in the art, the application must provide such information and that "it is not sufficient to provide it only through an expert's declaration." The present factual pattern is clearly distinct from that of Buchner in that the PTO has conceded in this record that the administration of cells was known in the medical art at the time of the present invention (page 22, first paragraph, of the Final Rejection of September 22, 2006). It is further

established in this record that the compositions (stem cells such as bone marrow stem cells), implantation apparatus (hypodermic needle) and treatment methods disclosed in the specification were well-known in the medical art. Contrary to the PTO's position, Appellant's evidence of enablement is supported by more than Declarants' conclusory statements. Declarants identify and rely upon facts, i.e., specific portions of the disclosure in the instant specification which support their conclusions that one skilled in the art would be able to make and use the claimed invention. Declarants' reading and understanding of the identified portions of the specification mentioned in Paragraph 5 of the Fourth Supplemental Declaration of Dr. Heuser and in the Third Supplemental Declaration of Dr. Lorincz, compels a conclusion that Dr. Elia was in possession the concept of implanting bone marrow stem cells and growing arteries and cardiac muscle in the heart of a human patient.

Other than stating that the PTO's position is set forth at pages 38-42 of the Examiner's Answer of January 24, 2008, the PTO has not critically addressed the probative value of Appellant's evidence. The PTO on aforesaid pages 38-42 has not specifically challenged Appellant's evidence and, rather, has set forth mere generalities. For example, it is not clear whether the PTO continues to assert that there are no post-filing publications in the record that show the growth of arteries. If this is the position of the PTO, it appears to be at odds with the PTO's present reliance upon Isner '887 and Kornowski. By failing to articulate adequate reasons to rebut the Declarations of Drs. Heuser and Lorincz, the PTO "failed to consider the totality of the record for the purpose of issuing a final rejection and thus erred as a matter of law." In re Alton, 76 F.3d 1168, 37 USPQ2d 1578 (Fed.Cir. 1996). It is trite law that the PTO must consider the

probative value of such evidence *vis-à-vis* any asserted *prima facie* case. See In re Oetiker, at 1445, 24 USPQ 2d at 1444. In re Keller, 642 F.2d 413, 208 USPQ 871, (CCPA 1981). In the absence of critical analysis, the PTO appears to be relying solely upon its opinion rather than assessing weight to the objective evidence proffered in the Declarations. PTO Examiners, not being skilled persons in the medical art, must give weight to these expert opinions rather than substitute the opinion of the PTO. See In re Neave, 370 F.2d 961, 152 USPQ 274, (CCPA 1967).

The PTO, at page 38, ¶50 of the Office Action, refers to the enablement factors enumerated by the court in In re Wands. The Board's attention is again respectfully directed to the In re Wands decision, which led to the grant of a patent. The Court found that the PTO's determination of nonenablement was unsupported by the evidence in the record. The Court further noted that the skill level in the art was high and that known materials were utilized in the practice of the invention in weighing the evidence. The instant fact situation is similar to that of In re Wands because the skill level is also high and known administration techniques and known materials are also utilized in the practice of the invention. In addition to such factual parallelism, Appellant provided expert objective evidence in the form of the Declarations of Drs. Heuser and Lorincz. These medical experts read relevant portions of the specification setting forth the generic invention and elected and non-elected species of such generic invention and determined that one skilled in the medical art, armed with the guidance and direction in the specification disclosures, would be enabled to practice the methods defined in the claims on appeal and to predictably anticipate the results defined therein without need for resorting to undue experimentation. Regarding complexity, the Board is again referred to

the spontaneous utterances mentioned above wherein the process was characterized as being simple by doctors skilled in the art. When the guidance and direction provided by Appellant's specification disclosure, the level of knowledge and the content of the prior art at the time of the invention as established in the record, the high level of skill in the art, and Appellant's declaration evidence are interpreted in a reasonable manner, analysis considering the factors in In re Wands compels a conclusion that undue experimentation would not be required to practice the invention called for in the appealed claims.

Once the relevant materials and administration techniques set forth in Appellant's specification and those known in the art when the application was filed, are properly considered in their entirety, Appellant believes that there should be no question that one skilled in the medical art is enabled to make and use the claimed invention. This conclusion is reinforced by the fact that the materials and administration techniques, but not the inventive result, were well known when the instant application was filed.

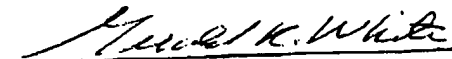
One final point remains regarding pages 2 and 3, ¶4 of the Office Action. The PTO states that where the grounds of rejection have been maintained from the Examiner's Answer dated January 24, 2008, "the specific arguments set forth in the Examiner's Answer...will not be repeated in the instant office action except where clarification may be deemed useful, omissions may be corrected..." In view of the fact that the PTO has not identified which grounds and arguments of said Examiner's Answer are incorporated in the rejections of the Office Action, Appellant reserves the right to answer in the form of a reply to any unenumerated grounds and arguments which may appear in any PTO response to the instant Brief.

CONCLUSION AND RELIEF SOUGHT

In view of the foregoing, Appellant urges the Board to reverse the outstanding rejection of claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 under 35 U.S.C. §112, first paragraph, and respectfully requests that the instant application be passed to issue.

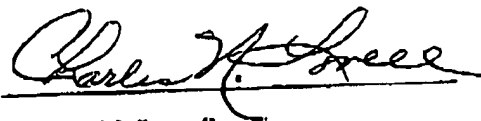
Respectfully submitted,

Dated: MAY 27, 2009



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Dated: May 27, 2009



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CLAIMS APPENDIX

Claims 236, 238, 239, 244, 247, 250, 251, 253, 257-263, 268-271, 280-285, and 288-290 are pending in the application, are under final rejection, are being appealed, and are listed below.

- | | |
|-----------|---|
| Claim 236 | A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart. |
| Claim 238 | The method of claim 236, further comprising repairing a dead portion of said heart. |
| Claim 239 | The method of claim 236, further comprising repairing a damaged portion of said heart. |
| Claim 244 | The method of claim 236, wherein said growth factor comprises a cell. |
| Claim 247 | The method of claim 236, wherein said growth factor is placed in said patient by injection. |
| Claim 250 | The method of claim 247, wherein said injection is intramuscular. |
| Claim 251 | The method of claim 236, wherein said growth factor is placed in said patient by a carrier. |
| Claim 253 | The method of claim 236, wherein said growth factor comprises a gene and a cell. |
| Claim 257 | The method of claim 236, wherein said growth factor is locally placed in said body. |

- Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.
- Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.
- Claim 260 The method of claim 244, wherein said growth factor is locally placed in said body.
- Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.
- Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.
- Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.
- Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.
- Claim 280 The method of claim 236 further comprising calculating blood flow through said newly grown artery.

- Claim 281 The method of claim 238 further comprising calculating blood flow through said newly grown artery.
- Claim 282 The method of claim 239 further comprising calculating blood flow through said newly grown artery.
- Claim 283 The method of claim 236 further comprising observing said newly grown artery.
- Claim 284 The method of claim 238 further comprising observing said newly grown artery.
- Claim 285 The method of claim 239 further comprising observing said newly grown artery.
- Claim 288 The method of claim 261, wherein said stem cells are harvested from bone marrow of said patient and are placed into the heart of the patient by injection.
- Claim 289 The method of claim 268, wherein said stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said dead portion.
- Claim 290 The method of claim 269, wherein said stem cells are harvested from the patient and are placed into the heart of the patient by injecting said stem cells at a site adjacent said damaged portion.

EVIDENCE APPENDIX

1. Office Action dated February 22, 2006 (page 6, lines 1-8) in co-pending application Serial No. 09/794,456 filed February 27, 2001.
2. Isner et al. U.S. Patent No. 5,980,887 cited by Appellant as Exhibit B to Reply Brief filed March 18, 2008.
3. Asahara et al. February 14, 1997 publication in Science entitled, "Isolation of Putative Progenitor Endothelial Cells for Angiogenesis," cited by Appellant as Reference ABA in 8th Supplemental Information Disclosure Statement filed September 24, 2008.
4. Nabel et al. U.S. Patent No. 5,328,470 cited by the Examiner in the November 28, 2003 Office Action.
5. Orlic et al. publication entitled, "Mobilized bone marrow cells repair the infarcted heart, improving function and survival," (August 28, 2001, PNAS USA, 98:10344-10349) and Orlic et al. publication entitled, "Bone marrow cells regenerate infarcted myocardium," (April 5, 2001, Nature, 410:701-705) cited on page 20 in Examiner's June 1, 2004, Final Office Action
6. Strauer et al. publication in Circulation entitled, "Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans" cited by Applicant as Exhibit E in Declaration of Dr. Richard Heuser filed June 17, 2003 (in connection with concurrently-filed Amendment).
7. Dohmann et al., 2005 publication in Circulation, 112:521-526, full copy of publication furnished in Examiner's Answer, November 28, 2007.
8. July 1, 2005 publication in The Journal of Invasive Cardiology, Vol. 17, entitled, "Tissue Engineering an Interventional Cardiology" cited by the Examiner in the October 2, 2008 Office Action (complete copy as furnished by Appellant and attached as Exhibit A to instant Appellant's Appeal Brief). An incomplete copy of this publication was furnished by the Examiner to Appellant (attached as Exhibit A1 to instant Appellant's Appeal Brief).
9. Murry et al. 1996 publication in J. Clin. Invest. entitled, "Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis" cited by the Examiner in the November 28, 2003 Office Action.

10. July 1, 2005 publication in The Journal of Invasive Cardiology, Vol. 17, entitled "Progenitor Cell Transplantation and Function following Myocardial Infarction" (author unknown) cited by the Examiner in the October 2, 2008 Office Action (complete copy as furnished by Appellant and attached as Exhibit B to instant Appellant's Appeal Brief). An incomplete copy of this publication was furnished by the Examiner to Appellant (attached as Exhibit B1 to instant Appellant's Appeal Brief).
11. Strauer et al. 2005 publication in Circulation entitled, "Regeneration of Human Infarcted Heart Muscle by Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease" cited by Applicant as Exhibit D in the Amendment filed November 21, 2005.
12. Alberts et al. 2002 publication in Molecular Biology of the Cell, 4th Ed., Chapter 17, cited by the Examiner in the October 2, 2008 Office Action.
13. 4th Supplemental Declaration of Dr. Heuser cited by Applicant as Exhibit A in the Amendment dated June 26, 2006.
14. 3rd Supplemental Declaration of Dr. Andrew E. Lorincz cited by Applicant as Exhibit B in the Amendment dated June 26, 2006.
15. 3rd Supplemental Declaration of Richard Heuser filed as Exhibit E of May 29, 2007 Amendment in Appellant's co-pending patent application Serial No. 10/179,589, and cited by Appellant as Exhibit C to Reply Brief filed March 18, 2008.
16. 2nd Supplemental Declaration of Andrew E. Lorincz filed as Exhibit D of May 29, 2007 Amendment in Appellant's co-pending patent application Serial No. 10/179,589, and cited by Appellant as Exhibit D to Reply Brief filed March 18, 2008.
17. Kornowski et al. U.S. Patent No. 7,097,832 cited by Appellant as Exhibit A to Reply Brief filed March 18, 2008.
18. 2004 Rabelink et al. publication in Arteriosclerosis, Thrombosis, and Vascular Biology, entitled, "Endothelial Progenitor Cells: More Than an Inflammatory Response?" cited by the Examiner in the October 2, 2008 Office Action.
19. Caplan 1991 publication in Journal of Orthopaedic Research, entitled, "Mesenchymal Stem Cells" cited by Applicant as Exhibit E in the Amendment filed June 26, 2006.
20. Declaration of Dr. Richard Heuser filed on June 17, 2003.
21. Supplemental Declaration of Dr. Heuser filed on February 17, 2004.

22. 2nd Supplemental Declaration of Dr. Heuser filed on dated July 30, 2004.
23. 3rd Supplemental Declaration of Dr. Heuser cited by Appellant as Exhibit I in the Appeal Brief filed June 13, 2005.
24. Declaration of Dr. Andrew E. Lorincz filed on June 17, 2003.
25. Supplemental Declaration of Dr. Andrew E. Lorincz filed on February 17, 2004.
26. 2nd Supplemental Declaration of Dr. Andrew E. Lorincz filed on July 30, 2004.
27. Final Office Action dated September 22, 2006 (page 22, first paragraph).

RELATED APPEALS AND INTERFERENCES APPENDIX

1. Co-pending Serial No. 09/794,456, filed February 27, 2001, in which Appellant's Brief was filed with the PTO on February 6, 2009;
2. Co-pending Serial No. 10/179,589, filed June 25, 2002, in which a Notice of Appeal was filed with the PTO on February 19, 2009; and
3. Co-pending Serial No. 09/064,000 filed April 21, 1998, in which a Notice of Appeal was mailed to the PTO on May 20, 2009.

EXHIBIT A

COMPLETE COPY OF

**July 1, 2005 publication in
The Journal of Invasive Cardiology, Vol. 17, entitled,
“Tissue Engineering an Interventional Cardiology”**

cited by the Examiner in the October 2, 2008 Office Action

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Issue Number:
7

author:

Speaker: David Holmes, MD
Moderator: Reginald Low, MD
Panel members: George Dangas, MD, Wolfgang Ritter, MD, Peter Gonschior, MD,
Brian Firth (Cordis Corporation)

George Dangas: There have been many attempts and many failures in this field due perhaps to the inherent tendency of interventional cardiologists to move quickly and work from assumptions, applying what may not yet be well understood. Gene therapy and engineered viruses (associated viruses, attenuated viruses, etc.) are examples of this. When it became clear that we were unable to identify the most appropriate and effective agent for angiogenesis, we looked toward the newly fashionable stem cell-based therapies. Even researchers make 3 million agents, and 2 of them turn out to be effective, that would be fine. On the other hand, perhaps the stem cells will produce 2 or 3 agents that work for angiogenesis, but at the same time, 1 or 2 other agents produce negative effects — the result being that the positive effect hoped for is not achieved. Thus, the interventional cardiology field must achieve more "crisp" results based on more "crisp" basic science, with better-established findings, in order to better understand what the targets are and pursue them in a more methodological manner. Our methodology needs to be evidence-based, as opposed to the focusing on the practicalities of how to achieve our aims. We need to scale down the in vivo applications and return to the laboratory.

David Holmes: There are a number of small, randomized trials currently under way, primarily in Europe. Perhaps some of our European colleagues here could discuss these trials. We are already in the middle of human trials before obtaining adequate scientific data about which specific cells to use, how many cells, when to deliver them, and how to deliver them. Is that a good thing? What if they fail? does that mean the approach is wrong? Or does it mean that we were doing it incorrectly?

Peter Gonschior: The good thing is that very robust cells are used based on solid, basic scientific data. That led to the application of a large variety of cells, which led to what appeared to be good data. The patient data, such as ejection fraction, however, are not terribly impressive. Ejection fraction improvement is not very significant, especially when you factor in the amount of energy wasted to achieve any clinical impact in the patients. More basic, relevant data are required to guide us toward the best approach.

Wolfgang Ritter: We have never used drug-eluting stents, so we wait to see what the cardiologist does.

David Holmes: From the industry standpoint, it would be nice to have a patentable product so that the product's unique design bearing the corporate name could be marketed. Given that God invented the progenitor cells and has a pretty strong patent on them, how do we "patent" a cell? For example, a bone marrow cell injected in the coronary artery — how do you design a device to do that? How do you make a living at that, since most any device could do that task?

Brian Firth: Let me come at this from a different angle. For some time now, Cordis has looked at what it already had as facilitating technology. We have been on the delivery side of the business, specifically with our Noga systems, the NogaStar®, the MyoStar™ injectable catheter, and so on. Thus, the mapping, definition and ability to deliver something in a very site-specific manner constitute the piece of the business Cordis has focused on. Having said that, in order to obtain 510-K FDA device approval, we must prove that it actually does something. Thus, Cordis is currently working on the area of autologous bone marrow with stem cells. Our interest is not in trying to figure out how to patent stem cells, which can't be done, but rather in the delivery of these cells, because we think that a more local delivery system would be better than a more general one. Cordis seeks to design a system, thus, that would deliver the cells that have been identified for their contractile properties to a site that has been defined as compromised.

Richard Heuser: These are expensive studies to conduct, thus, if the product is not patentable, it will not attract industry funding. In the case of the Bioheart study, how will this trial be conducted? Will sham cells be given, or no cells, or a small number of them? Also, we want to target the patient population that is not eligible for heart transplants. We have been talking about bone marrow cells as well. My understanding is that there is a very good possibility that these cells can be delivered intravenously with the same results. So how do we design, say, a skeletal muscle cell study that would actually end up garnering FDA approval for the therapy? And what about bone marrow — is it really necessary to go down the coronary arteries and go selectively into the myocardium?

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shown that when these different sorts of cells are delivered intravenously, they go to the lungs and have a "tremendous time," and they don't reach the myocardium. So while it makes perfect sense to use the intravenous approach, these cells are filtered out in the lungs and remain there. If those cells are active and produce cytokines, perhaps that's all we would want to use them for. Maybe these cells aren't the magic solution, and maybe we don't have a clue about this. Perhaps we can use these cells for the cytokines they produce systemically and they will cause other bone marrow cells to home in on the site of injury. But at the present, we just don't know enough about this process.

Patrick Whitlow: I just want to give you an update on Bioheart because of their underlying disease process, these patients are very prone to arrhythmia and sudden death. And theoretically, if you are adding islands of tissue in the left ventricle that is already damaged, these islands of tissue are not enervated in the same way as the surrounding tissue and the conduction properties aren't the same. You would theorize that this could set up re-entry circuits. Thus, ventricular arrhythmia presents an enormous problem in terms of conducting studies because many of those patients are going to die from their underlying disease. To detect if cell injection causes worsened arrhythmias will be very difficult, but a potentially serious problem. Therefore, the first v.s. clinical trial involves patients who already have defibrillators, and the number of patients will be small because of the need for defibrillators. The study should answer the question of whether this is arrhythmogenic — which Patrick Serruys believes is the case. Other researchers in France don't believe that injecting cells is arrhythmogenic. Who knows? It will take a long time and a lot of patients to arrive at the answer.

If a start-up company tries to make this therapy work, it will be very difficult for industry to actually fund the research from start to finish. We know from the animal studies that efficacy increases with higher doses of cell therapy, but we have yet to find what a potentially toxic dose is for the size of the island of cells that produce arrhythmias. I think that the bulk of this research will have to be federally funded. It's all very interesting work, and according to the animal models, it should work in humans.

David Holmes: Although the skeletal myoblasts appear to be arrhythmogenic, it appears to relate to engraftment properties. With true stem cells — whatever they are — it doesn't seem to be as problematic, whether because there have so far been very small numbers of patients, or whether indeed these stem cells are more pluripotent and engraft better, or whether they are more homogeneously distributed, and aren't just islands. It is early in this field, and I would echo what George said: in a field where so much rides on a product or technique, some of the trials are too premature because we often don't have the necessary solid scientific underpinnings before launching an important large trial. The biggest potential problem downstream to this approach is that if the product fails, we don't know for certain whether failure was due to the product's ineffectiveness or because we didn't know how to properly use it.

George Dangas: I would like to comment on interpreting the data from some of these early studies. I don't think we have the proper tools to accurately study the early results. The preliminary decision by the Rotterdam group was to implant defibrillators in all patients of the Bioheart study after two or three deaths occurred in one arm. Still, we haven't figured out whether it was actually the number of implants or if it was a patient substrate with a number of implants that caused the arrhythmogenicity. I think that any other study at this stage would produce statistical errors in both directions, which makes it very difficult to determine whether it was a failure of the agent, the liver system, or that the patients in the treatment arm were too sick and were going to die anyhow. That last explanation is a possibility because, due to ethical considerations, we usually enroll "no option" patients for these types of agents.

Richard Heuser: The Bioheart study involves a specific, patentable therapy which provides greater incentive to the company to see the project through to the end. One thing that always concerns me is determining what the endpoint will be. We all love to see those ejection fractions, but I think that the two main endpoints will likely be treadmill time (endurance) and objective findings of symptom relief. A third endpoint might be the number of hospitalizations for congestive heart failure. I agree that we have to conduct this study in some sort of randomized fashion. I think that the low-dose cells which we discussed will be a good way to do it. Also, since it's a very small number of patients being subjected to this very expensive therapy, I wonder if we could collect data on the patients before we commence therapy. In other words, we would assign the patient. We all know how long it takes to enroll patients in this trial; there's a lot of information to gather. During the six-month lead-in period, more data points could be obtained by looking at retrospective data on those individual patients. It won't be enough to see the ejection fraction increase, and there certainly won't be a reduction in mortality.

David Holmes: I think there will be a reduction in mortality rates and it will be the lead-in phase. For instance, all of the transplant centers have patient deaths while on the waiting list. This study will provide the same type of information. There may be other endpoints — viability of MR, for example. Whether viability with MR will be an "approvable" endpoint remains to be seen, however. We will need to be creative in terms of endpoints.

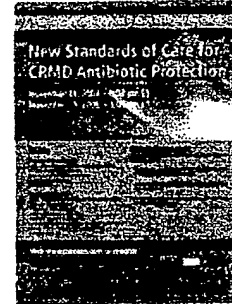
William O'Neill: I agree with you, George, in terms of the degree of our ignorance about the basic science in this area. My own feeling is that God — or nature — in His infinite wisdom, is a lot smarter than we will be for a few centuries yet in terms of the cascade of processes that actually allow a new cell to come in and regenerate. It is a little foolhardy to say that we should wait until we completely understand these processes before any clinical trials are launched. These early attempts are fine, as long as patients aren't harmed and as long as the patients are properly selected in terms of their ability to spontaneously improve function. As you said, pre-transplant patients will not improve function and there will likely be a big upside and very little downside for them. I would thus encourage conducting these small, mechanistic trials as a means of enlightening us as to where we stand and where we must go. Finally, when we change from the basic experiments to human trials, we are dealing with patients who are on all types of medications. Do ACE inhibitors, calcium channel blockers, beta-blockers and nitrates alter, improve or decrease the ability of cells to regenerate? We simply don't know the answer to this question. I do believe that we face a long process of trial and error, and will make small advances along the way.

David Holmes: I think that view is correct, provided that if the small trials are negative, we don't then abandon the field and decide that the therapy doesn't work. It seemed to be the case with some of the gene therapy trials where incredible hype was followed by randomized trials that produced negative results, setting the field back significantly. I think that well-designed studies aimed at identifying mechanisms will be terribly important for the field.

Brian Firth: In terms of endpoints, I believe that this falls under the same rules as most of the heart failure studies. The FDA wants to see that therapies designed for patients with heart failure or impending heart failure don't increase mortality while improving other parameters. Thus, researchers don't have to prove that the therapy improves survival rates, but they do have to prove that it doesn't adversely affect survival. That was the big lesson learned from the inotropic therapy studies.

Thomas McManara: What has been the progress and/or expectations with other critical organs — namely, the liver and the kidneys? Has work been done in this area?

David Holmes: I think work has been done, particularly on the liver, partly because it can regenerate. We tend to think that heart cells will repair what has been a problem, and I don't know if they will wildly proliferate and make a totally new heart, liver cells can do. You need to understand that I'm not exactly



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**July 1, 2005 publication in
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Journal of Invasive Cardiology

Tissue Engineering and Interventional Cardiology

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

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Issue Number:

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author:

Speaker: David Holmes, MD

Moderator: Reginald Low, MD

Panel members: George Dangas, MD, Wolfgang Ritter, MD, Peter Gonschior, MD,
Brian Firth (Cordis Corporation)

George Dangas: There have been many attempts and many failures in this field due perhaps to the inherent tendency of interventional cardiologists to move quickly and work from assumptions, applying what may not yet be well understood. Gene therapy and engineered viruses (associated viruses, attenuated viruses, etc.) are examples of this. When it became clear that we were unable to identify the most appropriate and effective agent for angiogenesis, we looked toward the newly fashionable stem cell-based therapies. Even researchers make 3 million agents, and 2 of them turn out to be effective, that would be fine. On the other hand, perhaps the stem cells will produce 2 or 3 agents that work for angiogenesis, but at the same time, 1 or 2 other agents produce negative effects — the result being that the positive effect hoped for is not achieved.

Thus, the interventional cardiology field must achieve more "crisp" results based on more "crisp" basic science, with better-established findings, in order to better understand what the targets are and pursue them in a more methodological manner. Our methodology needs to be evidence-based, as opposed to the focusing on the practicalities of how to achieve our aims. We need to scale down the in vivo applications and return to the laboratory.

David Holmes: There are a number of small, randomized trials currently under way, primarily in Europe. Perhaps some of our European colleagues here could discuss these trials. We are already in the middle of human trials before obtaining adequate scientific data about which specific cells to use, how many cells, when to deliver them, and how to deliver them. Is that a good thing? What if they fail? does that mean the approach is wrong? Or does it mean that we were doing it incorrectly?

Peter Gonschior: The good thing is that very robust cells are used based on solid, basic scientific data. That led to the application of a large variety of cells, which led to what appeared to be good data. The patient data, such as ejection fraction, however, are not terribly impressive. Ejection fraction improvement is not very significant, especially when you factor in the amount of energy wasted to achieve any clinical impact in the patients. More basic, relevant data are required to guide us toward the best approach.

Wolfgang Ritter: We have never used drug-eluting stents, so we wait to see what the cardiologist does.

David Holmes: From the industry standpoint, it would be nice to have a patentable product so that the product's unique design bearing the corporate name could be marketed. Given that God invented the progenitor cells and has a pretty strong patent on them, how do we "patent" a cell? For example, a bone marrow cell injected in the coronary artery — how do you design a device to do that? How do you make a living at that, since most any device could do that task?

Brian Firth: Let me come at this from a different angle. For some time now, Cordis has looked at what it already had as facilitating technology. We have been on the delivery side of the business, specifically with our Noga systems, the NogaStar®, the MyoStar™ injectable catheter, and so on. Thus, the mapping, definition and ability to deliver something in a very site-specific manner constitute the piece of the business Cordis has focused on. Having said that, in order to obtain 510-K FDA device approval, we must prove that it actually does something. Thus, Cordis is currently working on the area of autologous bone marrow with stem cells. Our interest is not in trying to figure out how to patent stem cells, which can't be done, but rather in the delivery of these cells, because we think that a more local delivery system would be better than a more general one. Cordis seeks to design a system, thus, that would deliver the cells that have been identified for their contractile properties to a site that has been defined as compromised.

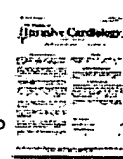
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Progenitor Cell Transplantation and Function following Myocardial Infarction

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William O'Neill: I have a couple of questions for both of you. First, we are essentially talking about adult, autologous stem cells. As you all know, there is enormous controversy in the U.S. about the use of embryonic stem cells which we are currently prohibited from using. From a dispassionate, basic scientific approach, do you think there's an advantage to using embryonic stem cells, or will adult stem cells be sufficient in terms of their ability to replicate and cause myogenesis?

Peter Gonschior: After examining the data from experiences in other fields with vascular cells and their biological functions, I don't think there is a need for embryonic stem cells. The bone marrow cells appear to be very robust; even the non-randomized European trial data show that with a not-so-profound approach, it is possible to obtain positive effects. Due to the political scenery in Europe, I don't think it would be possible to conduct large clinical trials using embryonic stem cells.

Sigrd Nikol: I think we should first try to optimize the strategies using autologous cells due to the ethical concerns. Due to these restrictions, there is not currently much proof available that embryonic stem cell therapy is that much better or even less risky. We know that these cells can differentiate, which means that they could differentiate into undesired cells as well. Thus, given the legal restrictions at present, we should stick with what we can do and try to make the best of it.

William O'Neill: Another key issue that arises from a clinical standpoint is that of direct injection versus intra-arterial or intravenous infusion. Do we know yet which would be the most efficient way — if the appropriate cell were to be found — to deliver the cells for the most efficient myogenesis?

Peter Gonschior: Sigrd and I have a lot of experience with local drug delivery. Efficiency of local drug delivery is in fact very low. It would be very smart to just inject the cells intravenously. But one critical issue with all of these data is that delivery efficiency, whether systemic or local, is very low. In fact, it turned out that endocardial delivery of these cells was the most efficient, resulting in the largest number of injected cells — up to about 30 million.

William O'Neill: The problem I have is that the stem cells are very sensitive to hypoxia, they need an oxygenated environment in order to thrive. Thus, if these cells are injected into a core infarct area, it will likely be hypoperfused and the ambient oxygen tension in that area may not be sufficient to support those cells. Sigrd, your experience involved a permanent ligation, right?

Sigrd Nikol: Actually, there were two sets of animals, but I only showed you those with the permanent ligation because there are currently more data on them. We also have a reperfusion model from 30 minutes after opening up the vessel.

William O'Neill: Did you find in the reperfusion model that there was any more myogenesis efficiency?

Sigrd Nikol: We perform stem cell therapy via bone marrow stimulation (not cell injection). At the moment, we do not have sufficient data on the reperfusion model. The problem is that when genes or cells are injected into the vessels, why should they pass the endothelium and migrate into the tissue where they are needed? It is very unlikely to happen, unless there is injured tissue. And with the venous application, there are the first-pass effects in the lungs and liver on top of it. These findings are not very convincing in my opinion, including the data from the Strasser group which lacked a truly randomized control group. The problem with intramyocardial injections into infarcted areas is a real one, as you stated, in that there is no blood/oxygen supply and potentially arrhythmogenic foci are created. Also, there may not be a homogeneous distribution of cells — an issue that has already been discussed with regard to gene therapy for the myocardium. Specifically, the question had been raised regarding how homogeneous gene therapy in the myocardium can be achieved without creating arrhythmias. With stem cells, this may be an even more important issue, as has been demonstrated in the work by Menasche et al.

William O'Neill: It seems to me that a highway is needed to get the cells to the tissue, and that's why we have been considering autointusion or direct intracoronary infusion of the agents, because in the reperfusion model, at least if there's an intact vascular structure, then the environment will be ambient and oxygenated until the cells can engraft. But my question is: Why would those cells stay at that target site? Also, we are lacking basic scientific understanding of the signals that allow the stem cells to home in on that particular tissue. Since stem cells could potentially be coming in from the systemic circulation, we need to come up with a homing material that could be injected into the infarcted area and would allow the stem cells to accumulate there. We are moving forward in this field.

At the 2003 AHA meeting, data from the late-breaking BOOST trial were presented. It was, I think, a very well-conducted randomized trial involving 60 patients with placebo versus bone marrow infusion arms. The results showed a statistically significant increase in ejection fractions in the active treatment group compared with the placebo group. TOPCARE was a terrific study, but it was a sort of historical control study. Thus, we are beginning to accumulate data that suggest there is some merit to this approach. Let me turn this question over now to the other panelists. Matt Pollman, from Guidant Corporation, is on the panel. Matt, is there business potential in this field?

Matt Pollman: That's a great question, Bill. As a clinician and a scientist, this area is extremely

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provocative and intriguing, particularly the data from the BOOST and TOPCARE myocardial infarction trials. There is an aggressive movement to push these trials out into more randomized, controlled, multicenter arenas. The issue for a company such as Guidant is what we can bring to the table and whether there is business potential in this area. Confidentiality, of course, prevents me from saying too much here, but there certainly appear to be business opportunities for companies like ours. We believe that it will require a very safe delivery system that can be applied to a wide range of patients. The cells clearly need to be delivered at least very close to the target site in order to successfully do their job. In that light, an intracoronary infusion strategy raises questions about how to allow enough time for the cells to adhere, to transigrate, enter the tissue and do their job. There is room for improvement in the delivery systems, and that's what Guidant is looking into. The TOPCARE acute myocardial infarction study headed up by Andreas Zeiher in Frankfurt uses a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries. The patients are then allowed to be in an unperfused state for 3 minutes to allow the cells to do their job. Often, the limiting factor is that the patients complain of chest pain during the unperfused state. Thus, there are opportunities to improve the current infusion protocol.

William O'Neill: We have been looking at it in a two-prong fashion, with a lot of interest in basic science on one end, and in clinical trials on the other end. As for the United States, if bone marrow is taken from a patient and then re injected, the FDA considers it non-homologous use, even though it is not a drug or a commercial compound. The U.S. FDA regulations on stem cell use do not cover autologous use of these cells. Unfortunately, thus, this entire field has become embroiled in the embryonic stem cell and abortion debates. As a result, the FDA is incredibly cautious about allowing embryonic stem cell use. The FDA regulations state that if the stem cells are taken and not manipulated, and then re injected, it falls outside of the FDA's authority. At the 2003 TCT meeting, the FDA representative specifically stated that if any compound is taken from the body and re injected (i.e., spinal fluid) into an area where it doesn't normally need to be, then the FDA does have regulatory authority.

Also, the FDA insists on receiving a significant amount of data — and rightfully so — on basic safety issues such as clonability and other matters surrounding the infusion of these cells into the coronary arteries. I think it will be another year or two in the U.S. before the basic science data are available to allow clinical trials to proceed. The bulk of the basic scientific studies will be carried out in South America and Europe.

Having said that, we have a perfect opportunity right here to learn from our colleagues about where this field is headed internationally. I know that you, Alfredo, are very much in the midst of all of this research. Would you mind telling us what your group will be doing in terms of your randomized trial?

Alfredo Rodriguez: Thank you, Bill. We are just starting a randomized trial that will follow the rules of the TOPCARE MI trial. Our trial involves 40 patients, 20 in each arm. One patient arm receives autologous bone marrow injections. These are acute myocardial infarction patients from 3 to 12 hours after symptom onset; all patients receive percutaneous coronary intervention and stenting. After reperfusion, we randomize the patients on day-4. On day-5, we puncture the patient's iliac crest, and the next day, we infuse the drug in 10 ml of solution into the coronary arteries.

The patient undergoes angiography immediately following the PCA procedure. Global and regional ejection fractions are measured. An acute and 4-month dobutamine stress echocardiogram is then done, followed by an MRI and SPECT imaging.

Our institution has a very active bone marrow transplant team. The hematologists who serve on our trial's executive committee told us that it was not necessary to place this trial under the Argentinian equivalent of the FDA, because autologous bone marrow is not a drug; it's not foreign material to the body. Thus, our trial is approved by the local transplant agency. My concern involves legal problems that could arise. I would like to hear from Sigrid and the other European colleagues here if the ongoing clinical trials in Europe in this field are approved by their respective regulatory agencies, or if they are only approved as protocols by the local hospitals' scientific committees, with the patients of course providing informed, written consent.

Sigrid Nikol: According to the blood transfusion and federal drug laws, there are certain regulatory approvals needed, particularly if the doctor obtaining the cells is not the same doctor using them therapeutically. In this case, cells are considered a blood product and their use is regulated.

Alfredo Rodriguez: I do know that the TOPCARE trial did not have local German regulatory approval.

Richard Heuser: I assume that we're talking here about a normal 10 cc bone marrow aspirant — no filtering — just administering it down the coronary arteries. Is that correct? And then the balloon is inflated for 3 minutes or so to allow the cells to disperse? And several injections are given?

Alfredo Rodriguez: Yes, 3 or 4 injections are given.

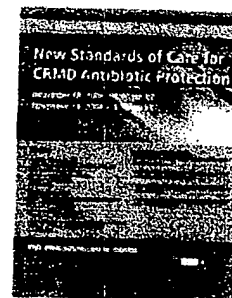
William O'Neill: This approach has generated controversy due to the fact that the bone marrow is unfractionated and thus contains fat, spicules, mesenchymal cells, and so on. Basically, the injection contains the "kitchen sink" and we hope that the right cells go to the right place and do the right thing. The other argument is that we know which cells we want, so we should just take them, filter them, grow them in media and replicate them, increase their efficiency, and then inject them. Those are the two schools of thought on the subject, but I can't tell you which is the correct one, because we might not have the right cells. It may be that the CD34 positive cells are not the right ones. In the TOPCARE study, they actually took both the peripheral cells and the cells obtained through leukapheresis, then identified them, segregated them, and grew them in a culture medium to increase their numbers.

In terms of FDA regulations, whenever you manipulate and produce cells, a commercial product results, and thus clearly falls under the FDA's purview. A regulatory "gray" area still exists in the U.S. when it comes to simply taking cells, leukapheresing them, removing the stem cells, and reinfusing them.

Richard Heuser: The first time I saw this technique presented by the group in Frankfurt, I was astonished at how simple it actually was. I am surprised that I didn't get into regulatory trouble myself about 5 or 6 years ago when I treated a patient in the middle of the night who tore a coronary artery. At that time, I had our home-made covered stents and some JoMed stents, but the vessel was 2.3 mm, and the patient was in cardiogenic shock. I had administered ReoPro and t-PA to this 70-year-old female patient. I just took some clot, combined it with a little of the prolamine, and it got to be enough of a slurry. I then put it down with a balloon, occluded the vessel, then re-opened it — and it was sealed. I then stented the vessel, and it was fine. But I find it hard to believe that if we administer these bone marrow cells to a patient with a huge infarct that we could get into trouble with the FDA. Some of these therapies make good sense for the individual patient, but more study data are needed.

William O'Neill: Let me pose a question to Paul Overlie, who has had extensive experience treating acute myocardial infarction patients for the past 20 years. Is there a need for this, Paul? The CADILLAC and recent myocardial infarction studies showed a 2% mortality rate and an ejection fraction mean of about 50%. How often does the situation arise that would warrant going to the trouble of doing bone marrow aspirates and leukapheresis on these patients?

Paul Overlie: The very high-risk, no-reflow patients might benefit from these therapies. Once these bone



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marrow cells are aspirated, is there some way to get the cytokines activated before injecting them, or concentrating them, so that the U.S. FDA would approve of the technique?

William O'Neill: Now that more studies have been conducted since the last time — about a year-and-a-half ago — that the FDA was approached about this, it's likely that the FDA will show more interest in allowing U.S. clinical trials to go forward. Phil, do you think there's an application for the genetic or stem cell repair approaches in the general vasculature — the aorta or peripheral vasculature?

Phillip Walker: I am a peripheral vascular surgeon, so I am definitely interested in myocardial repair to get our patients fit for intervention or following infarcts after intervention. There are a number of emerging areas where the approach might be helpful. Peter mentioned the single-center study on stem cell use for peripheral revascularization which involves an area where patients are nonreconstructible, particularly diabetics with renal failure.

I also wonder whether the no-reflow phenomenon — perhaps even in the setting of acute limb ischemia — might benefit from stem cell therapy. Stem cells may also be useful as an adjunct to tissue engineering. I work with a group who are developing a biological graft based on a peritoneal growth, which may be another useful area for the adjunctive use of stem cells. This therapy is being developed with the aim of improving the antithrombotic effects, which might also apply to prosthetic grafts that have been plagued by thrombotic problems when small diameters are involved.

Aortic repair in patients who have not yet developed sizable aneurysmal disease may be yet another area for stem cell therapy, but we need to learn how to identify these patients. We also need to learn to identify patients with small aneurysms, as stem cell repair might be useful in repairing and inhibiting the process in these patients.

Another viable area may be in the area of stroke and revascularization, as well as brain repair. This raises the issue of whether the mechanisms will be generic across all of those vascular beds, or whether differences exist, and whether the basic science needs to be worked out for the different areas.

I would like to ask about the issue of toxicity, particularly in diabetic patients in whom there may be an acceleration of diabetic retinopathy, tumorigenesis in the elderly patients, as well as plaque instability. Are these issues relevant?

William O'Neill: Perhaps because these bone marrow cells are pluripotent and have stimuli for differentiation, they will probably not be carcinogenic. And since they serve repair processes, it is unlikely that they will cause pathologic proliferation. Those are all critical questions that have plagued the field of gene therapy in which vectors were found that caused some cells to proliferate wildly. I think these cells will be safer, but we really won't know until a large number of patients are treated.

From our own acute myocardial infarction work, I presented a slide on the number of patients who present within 2 hours of symptom onset, and that number is about 5% of the U.S. acute myocardial infarction population — at least with the current standards. Perhaps with more novel, patient-directed approaches, this percentage could rise. After 2 hours, whether the patient is reperused or not, there will be a substantial amount of necrotic tissue and a large permanent infarct zone. If stem cell therapy could be safely applied, I believe that many patients could benefit in terms of improved regional function, making an akinetic anterior wall hypokinetic, or improving or preventing aneurysm formation.

Brian O'Murchu: Has the coronary sinus retrograde perfusion route been used for administration of these cells?

William O'Neill: Not that I am aware. There is one company that makes a device for access to the coronary sinus, and then needle injection into the myocardium. I think there may be some interest in using that as an access site rather than performing ventricular puncture.

Brian O'Murchu: I was just talking with my colleague, Alex Zapolanski, about whether the solution emerges from the ostia of the arterial coronaries during retrograde coronary perfusion, and of course it does. Thus, it would seem to provide the opportunity to "bathe" the myocardium through the use of a system that can be balloon-occluded, allowing perfusion to be maintained.

William O'Neill: That has been discussed, but I am not aware of any ongoing trials on that topic.

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EXHIBIT B1

INCOMPLETE COPY OF

**July 1, 2005 publication in
The Journal of Invasive Cardiology, Vol. 17, entitled
“Progenitor Cell Transplantation and Function following
Myocardial Infarction” (author unknown)**

cited by the Examiner in the October 2, 2008 Office Action

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Progenitor Cell Transplantation and Function following Myocardial Infarction

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:

7

William O'Neill: I have a couple of questions for both of you. First, we are essentially talking about adult, autologous stem cells. As you all know, there is enormous controversy in the U.S. about the use of embryonic stem cells which we are currently prohibited from using. From a dispassionate, basic scientific approach, do you think there's an advantage to using embryonic stem cells, or will adult stem cells be sufficient in terms of their ability to replicate and cause myogenesis?

Peter Gonschior: After examining the data from experiences in other fields with vascular cells and their biological functions, I don't think there is a need for embryonic stem cells. The bone marrow cells appear to be very robust; even the non-randomized European trial data show that with a not-so-profound approach, it is possible to obtain positive effects. Due to the political scenery in Europe, I don't think it would be possible to conduct large clinical trials using embryonic stem cells.

Sigrid Nikol: I think we should first try to optimize the strategies using autologous cells due to the ethical concerns. Due to these restrictions, there is not currently much proof available that embryonic stem cell therapy is that much better or even less risky. We know that these cells can differentiate, which means that they could differentiate into undesired cells as well. Thus, given the legal restrictions at present, we should stick with what we can do and try to make the best of it.

William O'Neill: Another key issue that arises from a clinical standpoint is that of direct injection versus intra-arterial or intravenous infusion. Do we know yet which would be the most efficient way — if the appropriate cell were to be found — to deliver the cells for the most efficient myogenesis?

Peter Gonschior: Sigrid and I have a lot of experience with local drug delivery. Efficiency of local drug delivery is in fact very low. It would be very smart to just inject the cells intravenously. But one critical issue with all of these data is that delivery efficiency, whether systemic or local, is very low. In fact, it turned out that endocardial delivery of these cells was the most efficient, resulting in the largest number of injected cells — up to about 30 million.

William O'Neill: The problem I have is that the stem cells are very sensitive to hypoxia; they need an oxygenated environment in order to thrive. Thus, if these cells are injected into a core infarct area, it will likely be hypoperfused and the ambient oxygen tension in that area may not be sufficient to support those cells. Sigrid, your experience involved a permanent ligation, right?

Sigrid Nikol: Actually, there were two sets of animals, but I only showed you those with the permanent ligation because there are currently more data on them. We also have a reperfusion model from 30 minutes after opening up the vessel.

William O'Neill: Did you find in the reperfusion model that there was any more myogenesis efficiency?

Sigrid Nikol: We perform stem cell therapy via bone marrow stimulation (not cell injection). At the moment, we do not have sufficient data on the reperfusion model. The problem is that when genes or cells are injected into the vessels, why should they pass the endothelium and migrate into the tissue where they are needed? It is very unlikely to happen, unless there is injured tissue. And with the venous application, there are the first-pass effects in the lungs and liver on top of it. These findings are not very convincing in my opinion, including the data from the Strauer group which lacked a truly randomized control group. The problem with intramyocardial injections into infarcted areas is a real one, as you stated, in that there is no blood/oxygen supply and potentially arrhythmogenic foci are created. Also, there may not be a homogeneous distribution of cells — an issue that has already been discussed with regard to gene therapy for the myocardium. Specifically, the question had been raised regarding how homogeneous gene therapy in the myocardium can be achieved without creating arrhythmias. With stem cells, this may be an even more important issue, as has been demonstrated in the work by Menasche et al.

William O'Neill: It seems to me that a highway is needed to get the cells to the tissue, and that's why we have been considering autotransfusion or direct intracoronary infusion of the agents, because in the reperfusion model, at least if there's an intact vascular structure, then the environment will be ambient and oxygenated until the cells can engraft. But my question is: Why would those cells stay at that target site? Also, we are lacking basic scientific understanding of the signals that allow the stem cells to home in on that particular tissue. Since stem cells could potentially be coming in from the systemic circulation, we need to come up with a homing material that could be injected into the infarcted area and would allow the stem cells to accumulate there. We are moving forward in this field.

At the 2003 AHA meeting, data from the late-breaking BOOST trial were presented. It was, I think, a very well-conducted randomized trial involving 60 patients with placebo versus bone marrow infusion arms. The results showed a statistically significant increase in ejection fractions in the active treatment group compared with the placebo group. TOPCARE was a terrific study, but it was a sort of historical control study. Thus, we are beginning to accumulate data that suggest there is some merit to this approach. Let me turn this question over now to the other panelists. Matt Pollman, from Guidant Corporation, is on the panel. Matt, is there business potential in this field?

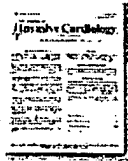
Matt Pollman: That's a great question, Bill. As a clinician and a scientist, this area is extremely provocative and intriguing, particularly the data from the BOOST and TOPCARE myocardial infarction trials. There is an aggressive movement to push these trials out into more randomized, controlled, multi-center arenas. The issue for a company such as Guidant is what we can bring to the table and whether there is business potential in this area. Confidentiality, of course, prevents me from saying too much here, but there certainly appear to be business opportunities for companies like ours. We believe that it will require a very safe delivery system that can be applied to a wide range of patients. The cells clearly need to be delivered at least very close to the target site in order to successfully do their job. In that light, an intracoronary infusion strategy raises questions about how to allow enough time for the cells to adhere, to transmigrate, enter the tissue and do their job. There is room for improvement in the delivery systems, and that's what Guidant is looking into. The TOPCARE acute myocardial infarction study headed up by Andreas Zeiher in Frankfurt uses a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries. The patients are then allowed to be in an unperfused state for 3 minutes to allow the cells to do their job. Often, the limiting factor is that the patients complain of chest pain during the unperfused state. Thus, there are opportunities to improve the current infusion protocol.

William O'Neill: We have been looking at it in a two-prong fashion, with a lot of interest in basic science on one end, and in clinical trials on the other end. As for the United States, if bone marrow is taken from a patient and then re-injected, the FDA considers it non-homologous use, even though it is not a drug or a commercial compound. The U.S. FDA regulations on stem cell use do not cover autologous use of these cells. Unfortunately, thus, this entire field has become embroiled in the embryonic stem cell and abortion debates. As a result, the FDA is incredibly cautious about allowing embryonic stem cell use. The FDA regulations state that if the stem cells are taken and not manipulated, and then re-injected, it falls outside of the FDA's authority. At the 2003 TCT meeting, the FDA representative specifically stated that if any

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EVIDENCE APPENDIX

EVIDENCE APPENDIX

ITEM NO. 1

**Office Action dated February 22, 2006 (page 6, lines 1-8)
in co-pending application Serial No. 09/794,456
filed February 27, 2001**

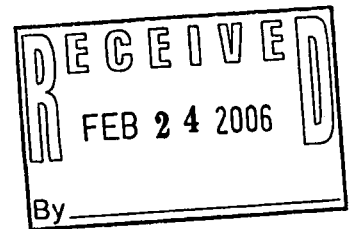


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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/794,456	02/27/2001	James P. Elia	1000-10-CO	4530
7590 02/22/2006				
Gerald K. White GERALD K. WHITE & ASSOCIATES, P.C. Suite 835 205 W. Randolph Street Chicago, IL 60606			EXAMINER KEMMERER, ELIZABETH	
			ART UNIT 1646	PAPER NUMBER
DATE MAILED: 02/22/2006				

Please find below and/or attached an Office communication concerning this application or proceeding.



Office Action Summary

Application No.

09/794,456

Applicant(s)

ELIA, JAMES P.

Examiner

Elizabeth C. Kemmerer, Ph.D.

Art Unit

1646

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 09 January 2006.
- 2a) ☐ This action is FINAL. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 7-40 is/are pending in the application.
- 4a) Of the above claim(s) 8-10 and 22-24 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 7, 11-21 and 25-40 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date 1/9/06.

- 4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: _____

DETAILED ACTION

Status of Application, Amendments, And/Or Claims

A request for continued examination under 37 CFR 1.114 was filed in this application after appeal to the Board of Patent Appeals and Interferences, but prior to a decision on the appeal. Since this application is eligible for continued examination under 37 CFR 1.114 and the fee set forth in 37 CFR 1.17(e) has been timely paid, the appeal has been withdrawn pursuant to 37 CFR 1.114 and prosecution in this application has been reopened pursuant to 37 CFR 1.114. Applicant's submission filed on 21 November 2005 has been entered.

Claims 1-6 are canceled. Claims 10-12 and 22-24 remain withdrawn from consideration as being directed to non-elected invention, for reasons of record. Claims 7, 11-21, and 25-40 are under examination.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Withdrawn Objections And/Or Rejections

The rejection of claims 7, 11-15, 18, 19, 21, 25-29, 32, and 33 as unpatentable over Murry et al. is *withdrawn* upon further consideration.

The rejection of claims 20 and 34 as unpatentable over Murry et al. in view of Nabel et al. is *withdrawn* upon further consideration.

35 U.S.C. § 112, First Paragraph – New Matter

Claims 16, 17, 20, 30, 31, and 34 remain rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The basis for this rejection is of record.

Applicant's arguments (pp. 11-13, amendment received 21 November 2005) have been fully considered but are not found to be persuasive for the following reasons.

As an initial matter, it is noted that Applicant incorrectly characterizes the instant rejection as an enablement rejection. The instant rejection is, and always has been, based on the written description component of 35 U.S.C. § 112, first paragraph, and not enablement. See p. 4 of the Office Action mailed 28 November 2003; p. 3 of the Office Action mailed 01 June 2004, and p. 2 of the Advisory Action mailed 26 November 2004. Applicant is reminded that *Vas-Cath Inc. v. Mahurkar*, 19USPQ2d 1111 (CAFC 1991) makes clear that the written description provision of 35 U.S.C. §112 is severable from its enablement provision (see page 1115).

Applicant points to p. 45 of the specification as describing injecting growth factors into a patient intravenously, intraluminally, or intramuscularly to promote growth of an artery, and applying genes or other genetic material with an

Art Unit: 1646

angioplasty balloon. This is not found to be persuasive because page 45, lines 13-16, of the specification reads as follows:

"VEGF **proteins** can be made in a lab and injected into a patient intravenously, intraluminally or intramuscularly to promote the growth of a new artery. Or, the **genes (or other genetic material)** can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method." (emphases added)

Clearly, this section of the specification is limited to use of proteins or nucleic acids (genes or genetic material). Regarding "intravenous" and "intraluminal" delivery, this section of the specification is limited to the suggestion of administering a protein. Nowhere else in the specification is it suggested that cells should be administered intravenously or intraluminally. Regarding angioplasty delivery, the second sentence quoted above is limited to the suggestion of administering genes or other genetic material by angioplasty balloon. The specification defines "growth factors" as comprising cells, but does not define "genetic material" as comprising cells. For example, p. 31, lines 11-13, of the specification states "...the genetic material comprises comparable artificially produced genes, or genes harvested from other human beings or animals." Page 32, lines 8-9 state "genetic material can comprise comparable artificially produced genes or genes removed from another animal or otherwise generated." Page 35, line 4 clearly distinguished between growth factors (defined as encompassing cells) and genetic material: "genetic material plus growth factor(s) are implanted..." Page 35, lines 12-14 states "Genetic material is well conserved in nature. The Drosophila eyeless gene (ey), the mouse small ey gene (pax-6), and the Aniridia gene in humans are all homologous." Page 36,

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lines 25-26 state "Genes control structure and function. A gene or a bit of genetic material may act as a master control gene..." Clearly, the specification uses "genetic material" as pertaining to nucleic acids such as genes. It is also noted that one skilled in the art would only interpret "assistance of a vector," recited in the same sentence that uses "genetic material," as only applying to nucleic acids (genes or RNA or cDNA, etc.).

Applicant points to Capon v. Eshhar v. Dudas, 03-1480-1481 (CAFC 2005) as controlling precedent that 112 does not require recitation in the specification of features already known by workers in the technological field to which the invention is directed. Applicant urges that the examiner mistakenly posited that generic inventions involving biochemical processes require a higher threshold for compliance with 112 because of a perception that success is not assured. Applicant argues that the Court in Capon observed that the USPTO must determine the sufficiency of support on a case-by-case basis given the state of the art at the time of the invention and in light of evidence of record. Applicant argues that the examiner has failed to address the generic concept that Applicant described – the concept of selecting a growth factor (herein the elected subgenus cells) and administering same into the body of a human patient using conventional methods and apparatus to achieve the goals of the claims. This is not found to be persuasive because the instant fact pattern is distinct from that in the case law cited by Applicant. Capon v. Eshhar, 76 USPQ2d 1078 (CAFC 2005) concerns whether or not claims to chimeric DNA molecules are adequately described by a generic description. The issue concerned written description of

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products, not method steps. The issue here is not whether or not workers in this technology already knew the features of the cells recited in the claims; rather, the issue is that the instant specification did not set forth contemplation of a method step wherein cells were administered intravenously, intraluminally, or via angioplasty. As discussed in the previous paragraph, the instant specification did not set forth contemplation of such method steps. The claims are being examined to the extent they read on the elected invention, administration of cells, and thus the generic concept of growth factor is not relevant. Furthermore,

MPEP § 2163.02 reads:

"An Applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. See Lockwood v. American Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention."

In the instant case, none of these criteria have been met. There was no reduction to practice, and the specification only refers to method steps involving proteins, genes and "genetic material," *but not cells*, as being useful in intravenous, intraluminal and angioplasty delivery. Therefore, the rejection is maintained.

35 U.S.C. § 112, First Paragraph – Enablement

Claims 7, 11-21, and 25-40 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The basis of this rejection is set forth at pp. 4-20 AND 29-41 of the Advisory Action mailed 26 November 2004, as well as at pp. 5-17 AND 24-28 of the Final Office Action mailed 01 June 2004.

Applicant's arguments (pp. 12-14, amendment received 21 November 2005) have been fully considered but are not found to be persuasive for the following reasons.

Applicant argues that evidence has been submitted to support enablement of the methods claimed in claims 16, 17, 30, and 31. Applicant points to Strauer 2002 and Strauer 2005 as repotting successful heart repair. Applicant indicates that there can be no "timing issue" that would require more than routine experimentation. This has been fully considered but is not found to be persuasive. Strauer (2002, Circulation 106:913, 1918) disclose balloon catheter injection of bone marrow cells to repair a dead portion of a heart. Claims 16, 17, 30, and 31 pertain to intravenous and intraluminal injection only, and thus Strauer is not particularly relevant. Although balloon catheter administration can be considered a species of the genus of intraluminal delivery methods, it is not commensurate in scope with the claims which read on delivery to the lumen of arteries, veins, intestines, heart chambers, lung, peritoneum, etc. Furthermore,

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Strauer's balloon catheter administration involved infusion of the cells by high-pressure injection directly into the necrotic area, to avoid the "wash-away" effect of standard intraluminal administration (Strauer, p. 1917, third paragraph of left column). It is noted that the specification as originally filed provides no guidance regarding high-pressure injection. Thus the post-filing date publication of Strauer cannot be relied upon to support enablement of the claims, as it uses methods which were not disclosed in the specification as originally filed. Additionally, Strauer specifically points out the shortcomings of intravenous administration of cells for heart therapy at p. 1917, second paragraph of the left column, where they state that "only a very small fraction of infused cells can reach the infarct region," "intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery," and "homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone." Thus, Strauer specifically provides evidence of non-enablement of the instant claims reciting intravenous administration of cells. "Strauer 2005" was not attached to the amendment. However, it was cited in a related application, and thus is made of record on the accompanying Notice of References cited. Strauer et al. (2005, J. Am. Coll. Cardiol. 46:1651-1658) used the same procedure as that in Strauer 2002, and thus also does not support enablement of claims 16, 17, 30, and 31 for the same reasons as discussed regarding Strauer 2002.

Regarding claims 7, 11-15, 18-21, 25-29, and 32-34, Applicant argues that the rejection must fail considering the totality of the evidence. Applicant urges

that the specification describes standard systems of identification as well as known procedures for selecting and isolating known cells (bone marrow stem cells) and known apparatus and methods for administering such cells to achieve the desired therapeutic result. Applicant indicates that specification describes specific materials and administration routes. This has been fully considered but is not found to be persuasive. Applicant does not point to any specific section of the specification as supporting these statements. The previous Office Actions have reviewed the specification's teachings and found that they do not provide enablement for the instant claims.

Applicant argues that the examiner has failed to consider the generic concept of selecting well-known appropriate cells and administering such cells using well-known methods and apparatus to grow muscles and arteries in a human patient's heart that do not occur in nature. Applicant urges that the examiner has failed to cite any evidence in the record showing this concept in the prior art. This has been considered but is not found to be persuasive in that it is a confusing argument. Is Applicant admitting that the claimed methods were so well known in the prior art that the specification need not have disclosed anything in addition to the prior art? Also, if the examiner had found evidence that the concept occurred in the prior art, then no enablement rejection would have been made. However, additional prior art rejections under 35 U.S.C. §§ 102 and 103 may have been made. Finally, 35 U.S.C. § 112, first paragraph, requires that the specification set forth what Applicant contemplates as the invention and provide sufficient disclosure so that the skilled artisan can make and use the claimed

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invention without undue experimentation. It is improper to pick and choose among unconnected sections of a specification in an attempt to capture another research group's post-filing date discoveries.

Finally, Applicant points to the Perin et al. trials as evidence that nothing more than routine experimentation was required to carry out the technique. Applicant characterizes Perin et al. as following Applicant's basic regimen. This has been fully considered but is not found to be persuasive. The Perin et al. evidence has already been considered. Perin refers to "transendocardial" injections of cells and "intramyocardial" injections of cells, both of which appear to be intramuscular forms of administration. Thus, Perin also cannot be used to support enablement of the rejected claims which recite intravenous or intraluminal administration of cells. Furthermore, the Perin et al. evidence submitted by Applicant is merely an Abstract which does not set forth any experimental details such that it can be ascertained whether or not Perin et al. used the same approach as that set forth in the claims corresponding to the same scope, or whether or not methods or materials other than those contemplated by Applicant were used. In other words, it cannot be ascertained whether or not Perin et al. constitutes evidence commensurate in scope with the claims.

Applicant submitted an article in an IDS dated 09 January 2006 (McDougall, 2005, Men's Health, pp. 164-171 and 194) to support enablement of the claimed invention. The reported information is based on an interview with Dr. Perin regarding his stem cell therapy approach for treating heart disease.

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Applicant quotes from the article that "it's like Miracle-Gro for your heart." Applicant characterizes the article as describing the procedure of injecting autologous bone marrow stem cells by catheter into the patient's heart as "astonishingly easy." Applicant argues that this submission is consistent with the expert evidence proffered by Drs. Heuser and Lorincz and supports Applicant's arguments that one skilled in the medical arts would have to resort to nothing more than routine experimentation to practice the described and claimed invention. Applicant urges that such submission further evinces the novel/pioneering nature of applicant's invention which uses old materials, old apparatus and old and well known methods to achieve a truly novel and miraculous result. This has been fully considered but is not found to be persuasive. The article submitted by Applicant is from "Men's Health" magazine, which is written for lay people, not those of ordinary skill in the art. Thus, any statements in the article regarding the ease or simplicity of the procedure are misleading. They may appear simple to the patient receiving the therapy; however, this has no bearing on how much research was involved in developing the techniques used. The research referred to in the article was that of Dr. Perin. So far, only a lay person's magazine article and an abstract have been submitted regarding Dr. Perin's trials. Such do not set forth the details relevant to the question of whether or not Dr. Perin's work supports enablement of the instant invention.

In view of the preponderance of the totality of the evidence, the enablement rejection is properly maintained.

35 U.S.C. § 112, Second Paragraph

Claims 13 and 27 remain rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The basis of this rejection is of record.

Applicant's arguments (pp. 7-11 of the amendment received 21 November 2005) have been fully considered but are not found to be persuasive for the following reasons.

Applicant reviews the recent finding in Phillips v. AWH Corporation (75 USPQ2d 1321) that claims are generally given their ordinary and customary meaning in the art, and that claims should be read in the context of the disclosure. Applicant argues that Phillips states that extrinsic evidence is less significant than the intrinsic record. Applicant points to the finding in Phillips that dictionary evidence can be useful, but such evidence is less reliable than specifications and prosecution histories. Applicant argues that the examiner should interpret the words "multifactorial and non-specific" in light of the specification, giving the words their ordinary meaning. Applicant argues that the examiner's interpretation is based on non-contextual sources places the terms out of context and do not enjoy the same weight of evidence as the specification. This has been fully considered but is not found to be persuasive. The examiner takes no issue with the general principles discussed in Phillips. The specification was the first place consulted by the examiner to breathe life and meaning into the

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term "multifactorial and non-specific" as applied to cells. As explained previously on the record, neither the specification nor the art provides an unambiguous definition for the term. Page 37 of the specification states, "Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated." The use of "such as" clearly implies that the term "multifactorial and non-specific cells" is intended to encompass cells other than stem cells and germinal cells. However, neither the specification nor the art disclose what these other cells are. In the absence of this information, the skilled artisan cannot determine the metes and bounds of the claims at issue. The functional portion of the definition, "...provide[s] the necessary in vivo and in vitro cascade of genetic material ..." makes no sense. What is a cascade of genetic material? Thus, the specification does not define these terms, and the metes and bounds of the claimed invention cannot be determined. A search of the prior art indicated that the relevant art also does not use the terms "multifactorial and non-specific" in connection with cells. See Appendix A, submitted as evidence, regarding a search done in the database Medline. The first result uses "multifactorial" to describe diseases. The second result uses "multifactorial" to describe a process. The third result uses "multifactorial" to describe a process. The fourth result uses "multifactorial" to describe analyses. The fifth result uses "multifactorial" to describe a process. The sixth result uses "multifactorial" to describe a study. Each of these usages is consistent with the examiner's position that the term "multifactorial," given its

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ordinary and customary usage in the art, is used to describe causes, effects and processes, not cells.

Applicant argues that the examiner's position is supported by a lack of search results regarding the terms followed by a series of suppositions and speculations regarding the meaning of the terms. Applicant characterizes the examiner's position as amounting to nothing more than opinion due to lack of evidence. Applicant indicates that while a chemist may interpret "multifactorial" to be limited to describing a process, one in the medical arts would not. Applicant urges that the term "factor" is well known in the medical art, and that "multifactorial" would be understood by one in the medical art to mean more than one factor. This has been fully considered but is not found to be persuasive. The rejection is supported by evidence. See discussion of the specification and attached search results. Applicant has provided no evidence that chemists and medical artisans would interpret "multifactorial" in different ways. Finally, Applicant's definition of "multifactorial" as meaning "more than one factor" makes no sense when applied to cells. What is a "more than one factor cell?"

Applicant refers to the fifth supplemental IDS as providing definitions. Applicant argues that the definitions are confirming evidence that the disputed terms are known and used properly in the specification, and that the IDS identifies the terms as adjectives. This has been fully considered but is not found to be persuasive. Regarding the dictionary definitions provided by the fifth supplemental IDS, the dictionary.net's definition of multifactorial is "involving or depending on several factors or causes (especially pertaining to a condition or

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disease resulting from the interaction of many genes)." This supports the rejection in that the term "multifactorial" is not used to describe cells. It is used to describe a cause (for example, of the disease) or an effect (for example, of the genes). Similarly, the dictionary.net's definition of nonspecific is "not caused by a specific agent; used also of staining in making microscope slides; 'nonspecific enteritis'" supports the rejection. "Nonspecific" is not used to describe cells. How can cells be "not caused by a specific agent?" The definition uses the term to describe causes (i.e., nonspecific enteritis is a disease caused by undefined factors). The selection of thesaurus words quoted by Appellant ("...undecided, undetermined, undifferentiated...") is also problematic. Cells can be in various stages of differentiation. For example, an embryonic stem cell would clearly be completely undifferentiated, as it can differentiate into any cell type. However, a promyelocyte is "undifferentiated" to an extent in that it can differentiate into a basophil, eosinophil, or neutrophil, whereas it cannot differentiate into any other cell type (e.g., keratinocytes, neural cells, muscle cells). The instant specification does not clarify whether such intermediate cells are encompassed by the term "multifactorial and non-specific."

Applicant provides definitions from Merriam Webster's Medline Plus

Medical Dictionary, namely:

Factor: (noun) a substance that functions in or promotes the function of a particular physiological process or bodily system.
Multifactorial: (adjective) having, involving, or produced by a variety of elements or causes.

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Applicant argues that "factor" means a substance such as a cell that promotes a particular physiological process, such as growth of an artery. Applicant argues that "multifactorial" is used to denote the quality of a cell when a variety of elements (factors) promote the growth of an artery. This has been fully considered but is not found to be persuasive. Applicant's definitions support the rejection. Applicant equates "factor" with "cell." Thus, substituting "cells" for "factors" in Applicant's second sentence, "multifactorial" is used to denote the quality of a cell when a variety of elements [cells] promote the growth of an artery. This simply makes no sense. Regarding the Merriam Webster's Medline Plus Medical Dictionary definition of multifactorial, what types of cells have, involve, or are produced by a variety of elements or causes?

Applicant argues that the terms were understood by those skilled in the art, pointing to the second supplemental declarations of Drs. Heuser and Lorincz. The second supplemental declarations of Drs. Heuser and Lorincz submitted under 37 CFR 1.132 are insufficient to overcome the rejection of claims 13 and 27 based upon 35 U.S.C. § 112, second paragraph because, although the declarations use the term "multifactorial and non-specific cells," they do not explain what cells are encompassed by the term. See section 7 of each of the Heuser and Lorincz second supplemental declarations. In view of the totality of the evidence of record, which includes the specification, prior art of record, and declarations submitted under 37 CFR 1.132, an unambiguous definition of the term "multifactorial and non-specific cells" has not been provided.

Applicant points to the two Strauer et al. publications. Specifically, Applicant points to p. 1656 of Strauer 2003 as stating that cardiac lesions are multifactorial. This has been fully considered but is not found to be persuasive because it supports the instant rejection. Strauer et al. 2003 was not attached to the response ; however, as it was cited in a related application, it has been cited on a Notice of References Cited and attached to this Office Action. Strauer et al. (2003, Circulation 107:929) uses the term "multifactorial" to describe a disease, not cells.

Applicant points to Strauer 2005 as stating that the regenerative potential of bone marrow derived stem cells may be explained by any of four mechanisms, and that "mechanisms" are further referred to as "factors." Applicant argues that the cells can be described as four-factor cells, i.e., multifactorial. Applicant concludes that the totality of the evidence indicates that the rejection should be withdrawn. Applicant also argues that "non-specific" is synonymous with "non-specialized." This has been fully considered but is not found to be persuasive. Strauer 2005 uses "four mechanisms" to describe "regenerative potential," not the cells *per se*. Even if Strauer 2005 could be tortuously construed as describing bone marrow stem cells as multifactorial, Strauer 2005 only discusses bone marrow stem cells. The specification already indicates that stem cells are exemplary of "multifactorial and non-specific" cells. The issue is what cells other than stem cells and germinal cells can be considered multifactorial and non-specific, given that the art does not apply these terms to cells.

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In view of the preponderance of the totality of the evidence, the rejection is maintained.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Elizabeth C. Kemmerer, Ph.D. whose telephone number is (571) 272-0874. The examiner can normally be reached on Monday through Thursday, 7:00 a.m. to 5:30 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Janet Andres, Ph.D. can be reached on (571) 272-0867. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Elizabeth C. Kemmerer

ECK

ELIZABETH KEMMERER
PRIMARY EXAMINER

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APPENDIX

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*File 155: Medline has resumed updating.

Set Items Description

? s (multifactorial (2N) cell?) and stem
12971 MULTIFACTORIAL
2761033 CELL?
66 MULTIFACTORIAL(2N)CELL?
143636 STEM
S1 6 (MULTIFACTORIAL (2N) CELL?) AND STEM
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18357667 PMID: 16020348

Current concepts in ocular surface reconstruction.

Dogru Murat; Tsubota Kazuo

Tokyo Dental College, Chiba, Japan.

Seminars in ophthalmology (United States) Apr-Jun 2005, 20 (2)

p75-93, ISSN 0882-0538 Journal Code: 8610759

Publishing Model Print

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

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Record type: MEDLINE; Completed

Diseases that affect the limbal %%%stem%%% %%%cells%%% are %%%multifactorial%%% and present with different stages of severity. The most important features to be considered in evaluating these patients include the degree of limbal %%%stem%%% cell loss, the extent of conjunctival disease, and the presence and etiology of ocular surface inflammation. Other important factors are tear film and eyelid abnormalities, keratinization of the ocular surface, laterality of the disease process, health and age of the patient. Careful consideration of all of these factors help tremendously in tailoring the most suitable method of treatment for each patient. The management of severe ocular surface disease has benefited from numerous advances in recent years. At one time, available techniques for visual rehabilitation consisted of superficial keratectomy, use of artificial tears, tarsorrhaphy as well as lamellar and penetrating keratoplasty. A lamellar or penetrating keratoplasty procedure resulted in a stable surface only for as long as the donor epithelium was present and once the epithelium sloughed off, the ocular surface failed due to conjunctivalization. The last few decades enjoyed the development and, especially, progress of new ocular surface reconstruction techniques such as amniotic membrane transplantation, limbal %%%stem%%% cell transplant procedures, transplantation of cultivated oral mucosal or limbal %%%stem%%% cell sheets. This review will briefly focus on the indications and methodology of each procedure and the currently available clinical data on the results of these procedures. (66 Refs.)

Record Date Created: 20050715

Record Date Completed: 20050818

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DIALOG(R)File 155:MEDLINE(R)

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15341984 PMID: 15145210

Robust conversion of marrow cells to skeletal muscle with formation of marrow-derived muscle %%%cell%%% colonies: a %%%multifactorial%%% process.

Abedi Mehrdad; Greer Deborah A; Colvin Gerald A; Demers Delia A; Dooner Mark S; Harpel Jasha A; Weier Heinz-Ulrich; Lambert Jean-Francois; Quesenberry P J

Roger Williams Medical Center, Department of Research, Providence, RI 02864, USA. mabedi@rwmc.org

Experimental hematology (Netherlands) May 2004, 32 (5) p426-34,

ISSN 0301-472X Journal Code: 0402313

Contract/Grant No.: 1P22-RR-18757-01; RR; NCRR; P01-DK-5022; DK; NIDDK; P01-HL-56920; HL; NHLBI; R01-DK-2742; DK; NIDDK; R01-DK-49650; DK; NIDDK

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

OBJECTIVE: Murine marrow cells are capable of repopulating skeletal muscle fibers. A point of concern has been the "robustness" of such conversions. We have investigated the impact of type of cell delivery, muscle injury, nature of delivered cell, and %%%stem%%% cell mobilizations on marrow-to-muscle conversion. METHODS: We transplanted green fluorescence protein (GFP)-transgenic marrow into irradiated C57BL/6 mice and then injured anterior tibialis muscle by cardiotoxin. One month after injury, sections were analyzed by standard and deconvolutional microscopy for expression of muscle and hematopoietic markers. RESULTS: Irradiation was essential to conversion, although whether by injury or induction of chimerism is not clear. Cardiotoxin- and, to a lesser extent, PBS-injected muscles showed significant number of GFP(+) muscle fibers, while uninjected muscles showed only rare GFP(+) cells. Marrow conversion to muscle was increased by two cycles of G-CSF mobilization and to a lesser extent by G-CSF and steel or GM-CSF. Transplantation of female GFP to male C57BL/6 and GFP to ROSA26 mice showed fusion of donor cells to recipient muscle. High numbers of donor-derived muscle colonies and up to 12% GFP(+) muscle cells were seen after mobilization or direct injection. These levels of donor muscle chimerism approach levels that could be clinically significant in developing strategies for the treatment of muscular dystrophies. CONCLUSION: In summary, the conversion of marrow to skeletal muscle cells is based on cell fusion and is critically dependent on injury. This conversion is also numerically significant and increases with mobilization.

Record Date Created: 20040517

Record Date Completed: 20040624

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DIALOG(R)File 155:MEDLINE(R)

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13036731 PMID: 11000981

Mobilization of peripheral blood progenitor cells for autografting: chemotherapy and G-CSF or GM-CSF.

Siena S; Bregni M; Gianni A M

The Falck Division of Medical Oncology, Ospedale Niguarda, Cai Granda, Milan, Italy.

Bailliere's best practice & research. Clinical haematology (ENGLAND)
Mar-Jun 1999, 12 (1-2) p27-39, ISSN 1521-6926 Journal Code: 100900679

Publishing Model Print

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

The mobilization of haematopoietic progenitor %%%cells%%% is a %%%multifactorial%%% process, still poorly understood at the molecular level. Mobilized haematopoietic progenitors, as defined by the expression of CD34 cell surface molecule, comprise heterogeneous subpopulations of cells committed to different haematopoietic lineages. Haematopoietic progenitors may be mobilized by chemotherapy alone, haematopoietic growth factors alone, or by chemotherapy plus haematopoietic growth factors. The choice of a mobilization regimen that allows an optimal yield of progenitors with a minimum number of leukaphereses should incorporate, in most patients, a disease-specific chemotherapeutic agent(s) plus a haematopoietic growth factor, to be continued until completion of harvest.
(76 Refs.)

Record Date Created: 20001019

Record Date Completed: 20001019

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DIALOG(R)File 155:MEDLINE(R)

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12278080 PMID: 9588003

Advances in hematopoietic %%%stem%%% cell culture.

Audet J; Zandstra P W; Eaves C J; Piret J M

Biotechnology Laboratory, University of British Columbia, Vancouver, Canada.

Current opinion in biotechnology (ENGLAND) Apr 1998, 9 (2) p146-51,
ISSN 0958-1669 Journal Code: 9100492

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Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Recent advances in our understanding of the earliest stages of hematopoietic cell differentiation, and how these may be manipulated under defined conditions in vitro, have set the stage for the development of robust bioprocess technology applicable to hematopoietic cells. Sensitive and specific assays now exist for measuring the frequency of hematopoietic %%%stem%%% cells with long-term in vivo repopulating activity from human as well as murine sources. The production of natural or engineered ligands through recombinant DNA and/or combinatorial chemistry strategies is providing new reagents for enhancing the productivity of hematopoietic %%%cell%%% cultures. %%%Multifactorial%%% and dose-response analyses have yielded new insight into the different types and concentrations of factors required to optimize the rate and the extent of amplification of specific subpopulations of primitive hematopoietic cells. In addition, the rate of cytokine depletion from the medium has also been found to be dependent on the types of cell present. The discovery of these cell-type-specific

parameters affecting cytokine concentrations and responses has introduced a new level of complexity into the design of optimized hematopoietic bioprocess systems. (49 Refs.)

Record Date Created: 19980608

Record Date Completed: 19980608

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DIALOG(R)File 155:MEDLINE(R)

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11563022 PMID: 8875201

Role of the basal cells in premalignant changes of the human prostate: a %%%stem%%% cell concept for the development of prostate cancer.

Bonkhoff H

Institute of Pathology, University of the Saarland, Homburg/Saar, Germany.

European urology (SWITZERLAND) 1996, 30 (2) p201-5, ISSN 0302-2838

Journal Code: 7512719

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

OBJECTIVES: Prostatic intraepithelial neoplasias (PIN) result from abnormal differentiation and proliferation processes within the prostatic epithelial cell system. Recent data indicate that basal cells are essentially involved in normal and abnormal growth patterns of the human prostate. **RESULTS:** The basal cell layer represents the proliferative compartment and most probably houses the prostatic %%%stem%%% cell population. Basal cells are targets of several regulatory factors including estrogens, androgens, epidermal growth factor and other nonsteroidal growth factors. During the malignant transformation of the prostatic epithelium (PIN), the basal cell layer loses its proliferative function which is transferred to secretory luminal cell types. These proliferative abnormalities are attended by severe regulatory disorders of the programmed cell death within the prostatic epithelial cell system. The Bcl-2 oncoprotein which blocks the programmed cell death in the proliferative compartment (basal cell layer) in normal conditions, extends to the secretory luminal cell types in high-grade PIN lesions. This, in turn, may increase the genetic instability of the dysplastic epithelium. During the process of tumor invasion, the transformed cells lose their basal cell-specific phenotype and acquire features of exocrine cell types which represent the major phenotype in common prostate cancer. At the point of stromal invasion, the transformed cells produce neoplastic basement membrane material which allows them to penetrate the extracellular matrix. **CONCLUSION:** These data provide theoretical bases for a %%%stem%%% cell concept in the development of prostate cancer and highlights the importance of basal %%%cells%%% in this %%%multifactorial%%% process.

Record Date Created: 19970116

Record Date Completed: 19970116

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DIALOG(R)File 155:MEDLINE(R)

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11537242 PMID: 8850230

[T-cell-rich B-cell lymphoma: multifactorial study of 4 cases]

Linfoma B rico en células T: estudio multifactorial de cuatro casos.

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PURPOSE: With the correlational study of four cases in several areas (clinic, morphoimmunological, ultrastructural and genetic) we try to valorate the still controversial entity known as T-cell rich B-cell lymphoma (TRBL), and establish some useful clues in order to settle down the differential diagnosis between TRBL, Hodgkin's disease (HD), and T-cell non-Hodgkin's lymphomas (TNHL). **PATIENTS AND METHODS:** Cases proceeded from Oncology Department, and had been firstly misdiagnosed either as HD (3 cases) or as TNHL (1 case). Biopsies were processed and stained in routine way, H&E, Giemsa and Wilder. Immunohistological study, using monoclonal antibodies against B-cells, T-cells, histiocytes, activation and proliferation markers, was also performed with avidin biotin peroxidase (ABC) method. Ultrastructural study was performed in three of the cases; two patients were studied by PCR and Southern blot. **RESULTS:** All of the cases showed a diffuse histological pattern, with variable fibrosis, and proliferation of venules and capillaries. Small lymphoid cells, being positive for CD3, were dominant. Large blastic cells, positive for CD20, some of them with a Sternberg-like appearance, could be found, in a spotty pattern. Histiocytes were abundant and positive to CD68. Proliferation index (Ki-67) ranged between 13 and 24.5% being the stain mainly positive for B-cells and in a certain extent, also for T-cells. Ultrastructural features were closer to those of the NHL than to the ones found in HD. Molecular study failed to prove any rearrangement. **CONCLUSIONS:** TRBL is a rare entity between B-cell NHL group. Diagnosis and differential diagnosis (mostly with HD and T-cell NHL) have to be properly made, because of the very distinct prognosis and therapy.

Record Date Created: 19961217

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**List of Patents and Publications For
Applicant's 6th Supplemental
Information Disclosure Statement
Page 1 of 1**



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Filing Date: February 27, 2001

Applicant: James P. Elia

Group No.: 1646

Examiner: Elizabeth Kemmerer

Reference Designation U.S. PATENT DOCUMENTS							
Examiner Initial		Document Number	Date	Name	Class	Sub-Class	Filing Date (if appropriate)

Foreign Patent Documents							
		Document Number	Date	Country	Class	Sub-Class	Translation Yes No

Other Art (including author, title, date, pertinent pages, etc.)		
<div style="font-family: cursive; font-size: 1.2em;">EOK</div>	AU	Men's Health, December 2005, McDougall, "The Greatest medical Revolution of the Century is about to begin"

Examiner <div style="font-family: cursive; font-size: 1.2em;">E. Kemmerer</div>	Date Considered <div style="font-family: cursive; font-size: 1.2em;">2/16/06</div>
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Notice of References Cited

Application/Control No.

09/794,456

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Examiner

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NON-PATENT DOCUMENTS

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*	V	Strauer et al., 2003, Circulation 107:929.
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EVIDENCE APPENDIX

ITEM NO. 2

Isner U.S. Patent No. 5,980,887

[54] **METHODS FOR ENHANCING ANGIOGENESIS WITH ENDOTHELIAL PROGENITOR CELLS**

[75] Inventors: Jeffrey M. Isner, Weston; Takayuki Asahara, Arlington, both of Mass.

[73] Assignee: St. Elizabeth's Medical Center of Boston, Boston, Mass.

[21] Appl. No.: 08/744,882

[22] Filed: Nov. 8, 1996

[51] Int. Cl.⁶ A61K 35/12; A61K 48/00; A61K 38/18; A61K 38/19

[52] U.S. Cl. 424/93.7; 424/85.1; 424/85.2; 514/8; 514/44

[58] Field of Search 424/93.7, 85.4, 424/85.2; 435/325, 375; 514/2, 8, 44; 530/351; 53/23.5

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Primary Examiner—Christina Y. Chan

Assistant Examiner—Phillip Gambel

Attorney, Agent, or Firm—David G. Conlin; David S. Resnick; Dike, Bronstein, Roberts & Cushman, LLP

[57] **ABSTRACT**

In accordance with the present invention, EC progenitors can be used in a method for regulating angiogenesis, i.e., enhancing or inhibiting blood vessel formation, in a selected patient and in some preferred embodiments for targeting specific locations. For example, the EC progenitors can be used to enhance angiogenesis or to deliver an angiogenesis modulator, e.g. anti- or pro-angiogenic agents, respectively to sites of pathologic or utilitarian angiogenesis. Additionally, in another embodiment, EC progenitors can be used to induce reendothelialization of an injured blood vessel, and thus reduce restenosis by indirectly inhibiting smooth muscle cell proliferation.

11 Claims, 7 Drawing Sheets

FIG. 1A



FIG. 1B

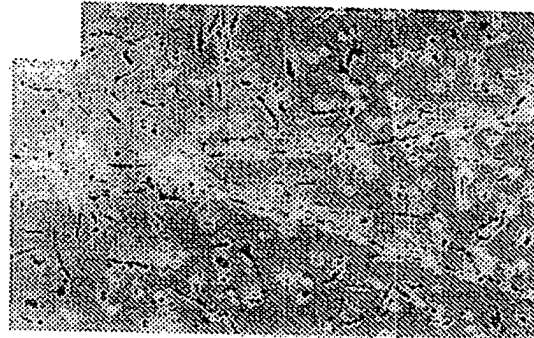


FIG. 1C

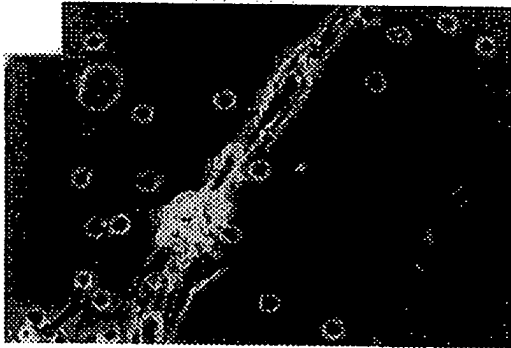


FIG. 1D

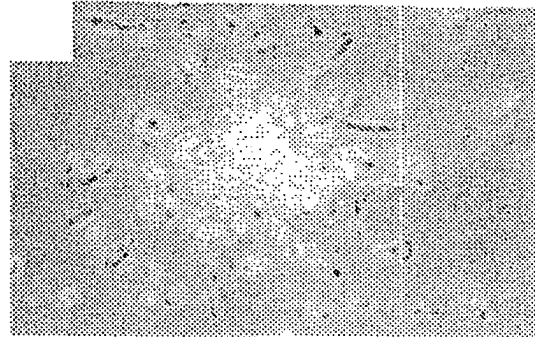


FIG. 1E

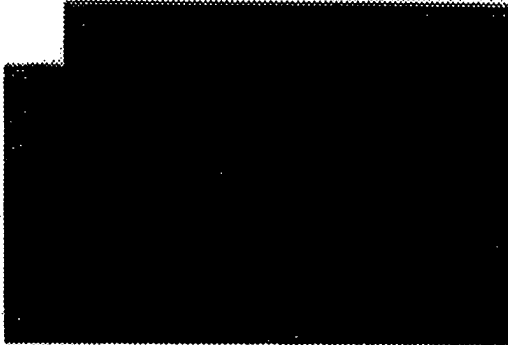


FIG. 1F

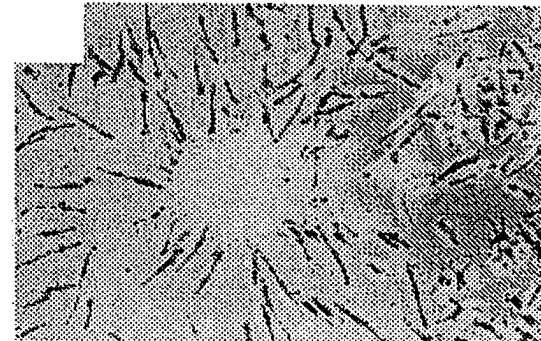


FIG. 1G



FIG. 2

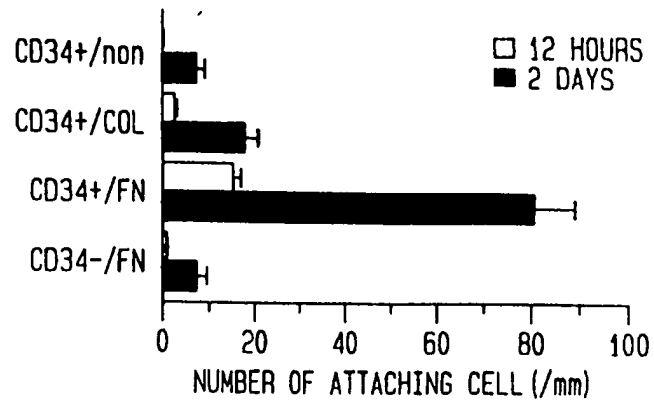


FIG. 5

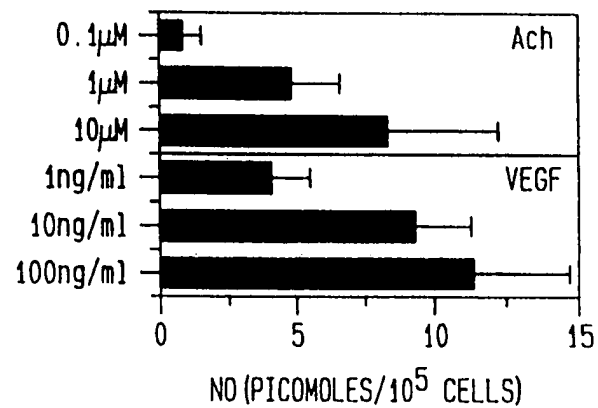


FIG. 7

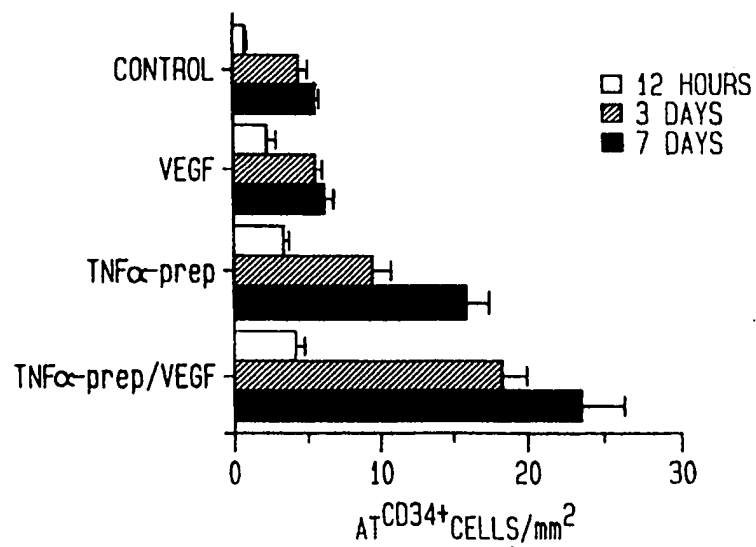


FIG. 3

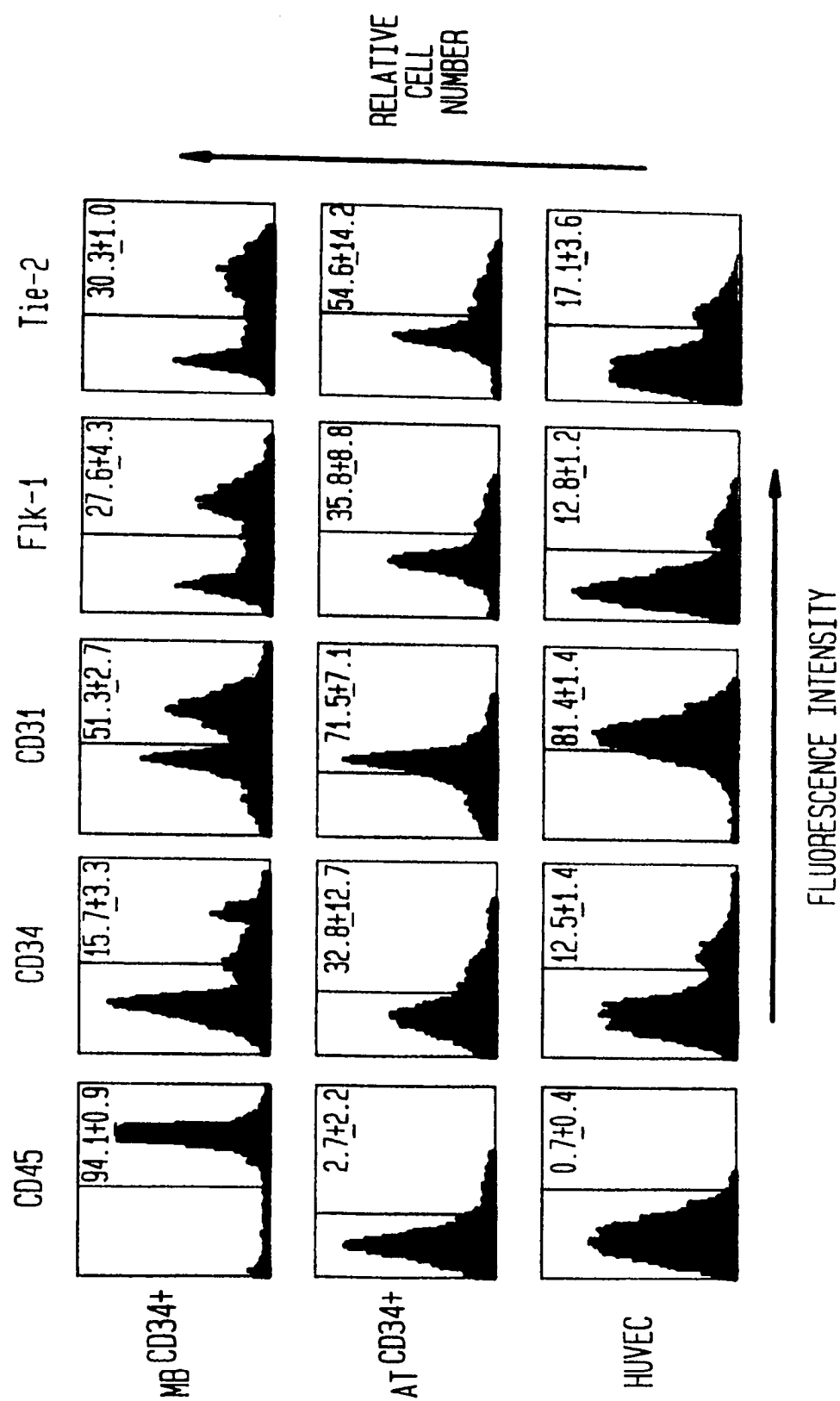


FIG. 4

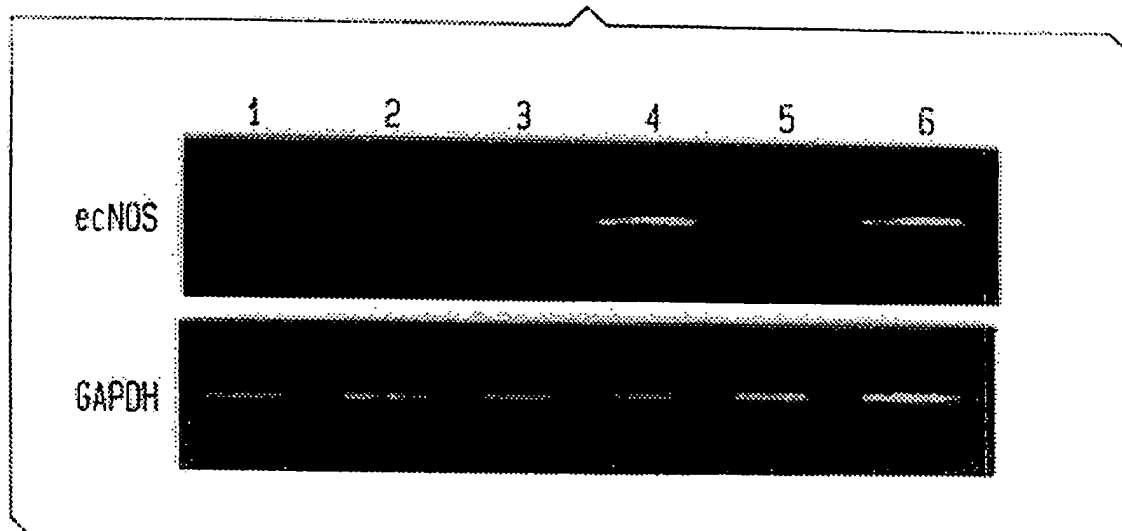


FIG. 9

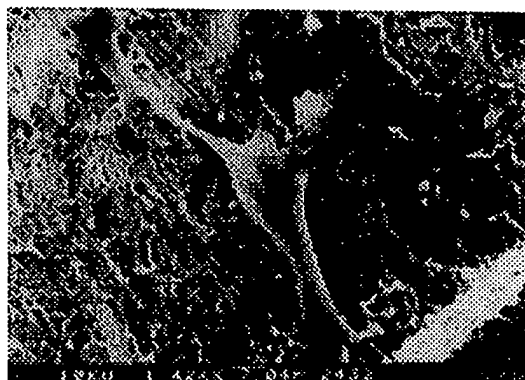


FIG. 6A



FIG. 6B

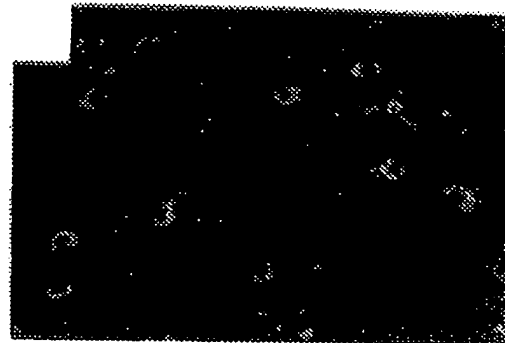


FIG. 6C

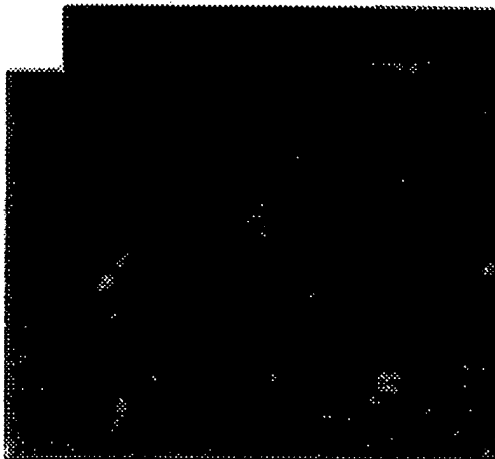
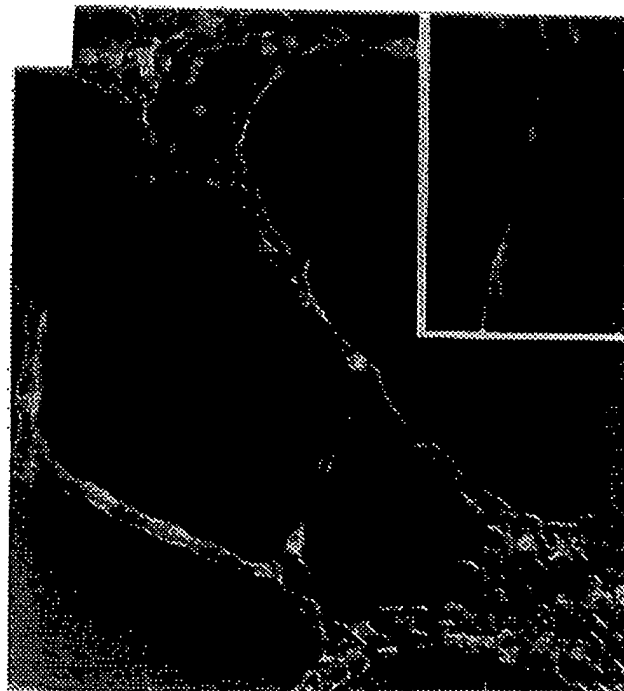


FIG. 6D



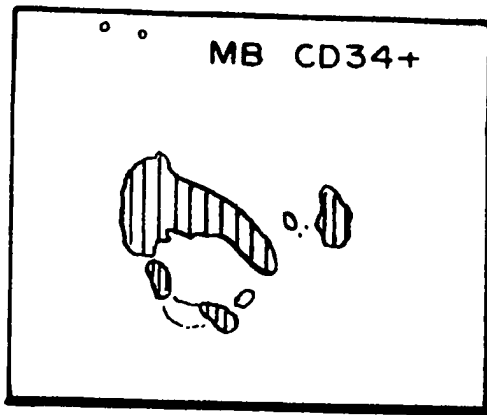


FIG. 8A

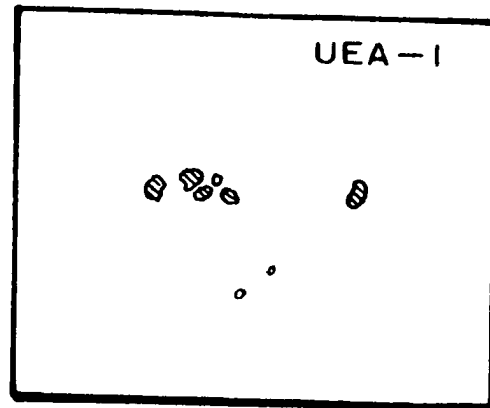


FIG. 8B

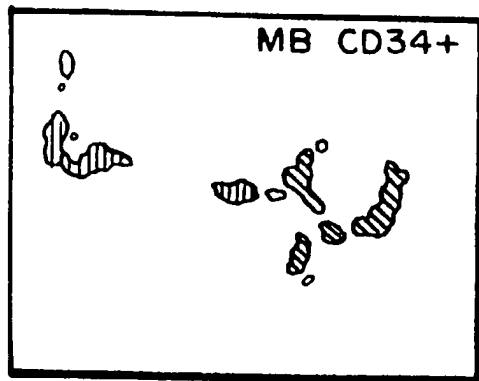


FIG. 8C

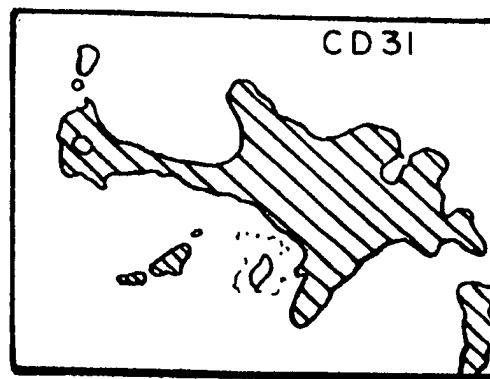


FIG. 8D

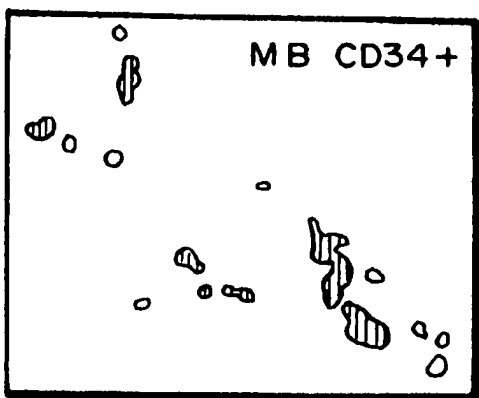


FIG. 8E

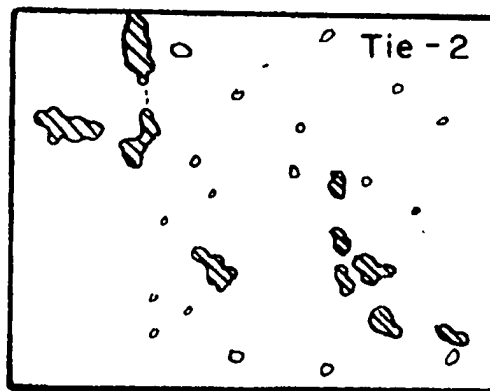


FIG. 8F

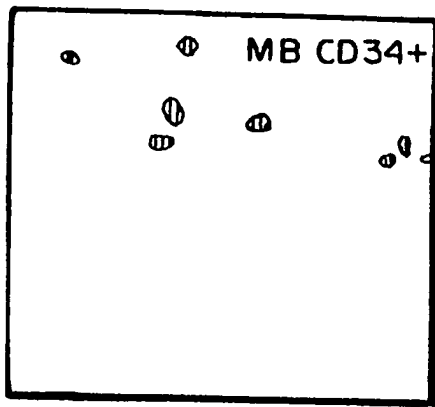


FIG. 8G

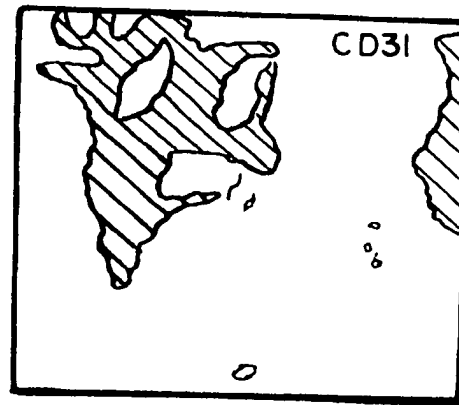


FIG. 8H

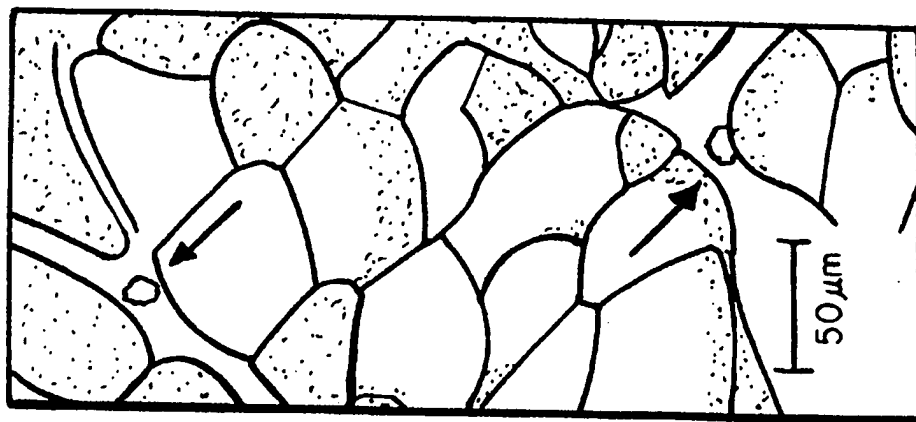


FIG. 8I

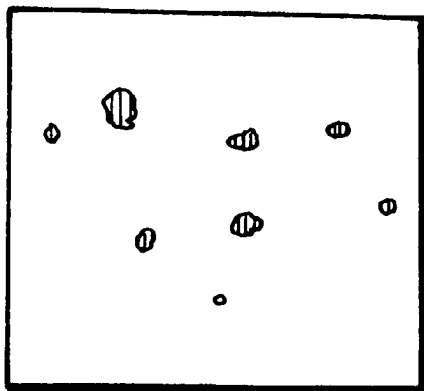


FIG. 8J

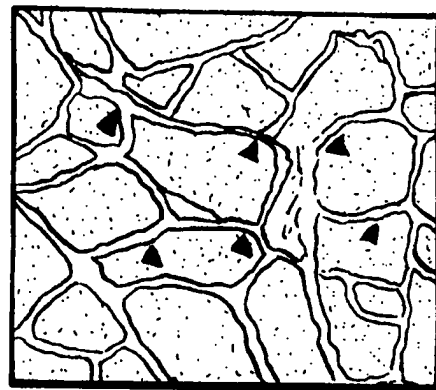


FIG. 8K

METHODS FOR ENHANCING ANGIOGENESIS WITH ENDOTHELIAL PROGENITOR CELLS

BACKGROUND OF THE INVENTION

Blood vessels are the means by which oxygen and nutrients are supplied to living tissues and waste products removed from living tissue. Angiogenesis is the process by which new blood vessels are formed, as reviewed, for example, by Folkman and Shing, *J. Biol. Chem.* 267 (16), 10931-10934 (1992). Thus angiogenesis is a critical process. It is essential in reproduction, development and wound repair. However, inappropriate angiogenesis can have severe consequences. For example, it is only after many solid tumors are vascularized as a result of angiogenesis that the tumors begin to grow rapidly and metastasize. Because angiogenesis is so critical to these functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed to begin with the degradation of the basement membrane by proteases secreted from endothelial cells (EC) activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The cells migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space, then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane.

In the adults, the proliferation rate of endothelial cells is typically low compared to other cell types in the body. The turnover time of these cells can exceed one thousand days. Physiological exceptions in which angiogenesis results in rapid proliferation occurs under tight regulation are found in the female reproduction system and during wound healing.

The rate of angiogenesis involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels. Abnormal angiogenesis occurs when the body loses its control of angiogenesis, resulting in either excessive or insufficient blood vessel growth. For instance, conditions such as ulcers, strokes, and heart attacks may result from the absence of angiogenesis normally required for natural healing. On the contrary, excessive blood vessel proliferation may favor tumor growth and spreading, blindness, psoriasis and rheumatoid arthritis.

The therapeutic implications of angiogenic growth factors were first described by Folkman and colleagues over two decades ago (Folkman, *N. Engl. J. Med.*, 285:1182-1186 (1971)). Thus, there are instances where a greater degree of angiogenesis is desirable—wound and ulcer healing. Recent investigations have established the feasibility of using recombinant angiogenic growth factors, such as fibroblast growth factor (FGF) family (Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992) and Baffour, et al., *J Vasc Surg*, 16:181-91 (1992)), endothelial cell growth factor (ECGF)(Pu, et al., *J Surg Res*, 54:575-83 (1993)), and more recently, vascular endothelial growth factor (VEGF) to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia (Takeshita, et al., *Circulation*, 90:228-234 (1994) and Takeshita, et al., *J Clin Invest*, 93:662-70 (1994)).

Conversely, there are also instances, where inhibition of angiogenesis is desirable. For example, many diseases are driven by persistent unregulated angiogenesis. In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries invade the vitreous, bleed, and cause blindness. Ocular neovascularization is the most common cause of blindness. Tumor growth and

metastasis are angiogenesis-dependent. A tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow.

The current treatment of these diseases is inadequate. Agents which prevent continued angiogenesis, e.g. drugs (TNP-470), monoclonal antibodies and antisense nucleic acids, are currently being tested. However, new agents that inhibit angiogenesis are need.

Recently, the feasibility of gene therapy for modulating angiogenesis has been demonstrated. For example, promoting angiogenesis in the treatment of ischemia was demonstrated in a rabbit model and in human clinical trials with VEGF using a Hydrogel-coated angioplasty balloon as the gene delivery system. Successful transfer and sustained expression of the VEGF gene in the vessel wall subsequently augmented neovascularization in the ischemic limb (Takeshita, et al., *Laboratory Investigation*, 75:487-502 (1996); Isner, et al., *Lancet*, 348:370 (1996)). In addition, it has been demonstrated that direct intramuscular injection of DNA encoding VEGF into ischemic tissue induces angiogenesis, providing the ischemic tissue with increased blood vessels (U.S. Ser. No. 08/545,998; Tsurumi et al., *Circulation*, In Press).

Alternative methods for regulating angiogenesis are still desirable for a number of reasons. For example, it is believed that native endothelial cell (EC) number and/or viability decreases over time. Thus, in certain patient populations, e.g., the elderly, the resident population of ECs that is competent to respond to administered angiogenic cytokines may be limited.

Moreover, while agents promoting or inhibiting angiogenesis may be useful at one location, they may be undesirable at another location. Thus, means to more precisely regulate angiogenesis at a given location are desirable.

SUMMARY OF THE INVENTION

We have now discovered that by using techniques similar to those employed for HSCs, EC progenitors can be isolated from circulating blood. In vitro, these cells differentiate into ECs. Indeed, one can use a multipotentiate undifferentiated cell as long as it is still capable of becoming an EC, if one adds appropriate agents to result in it differentiating into an EC.

We have also discovered that in vivo, heterologous, homologous, and autologous EC progenitor grafts incorporate into sites of active angiogenesis or blood vessel injury, i.e. they selectively migrate to such locations. This observation was surprising. Accordingly, one can target such sites by the present invention.

The present invention provides a method for regulating angiogenesis in a selected patient in need of a change in the rate of angiogenesis at a selected site. The change in angiogenesis necessary may be reduction or enhancement of angiogenesis. This is determined by the disorder to be treated. In accordance with the method of the present invention, an effective amount of an endothelial progenitor cell or modified version thereof to accomplish the desired result is administered to the patient.

In order to reduce undesired angiogenesis, for example, in the treatment of diseases such as rheumatoid arthritis, psoriasis, ocular neovascularization, diabetic retinopathy, neovascular glaucoma, angiogenesis-dependent tumors and tumor metastasis, a modified endothelial cell, having been modified to contain a compound that inhibits angiogenesis, e.g., a cytotoxic compound or angiogenesis inhibitor, can be administered.

To enhance angiogenesis, for example in the treatment of cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia, endothelial progenitor cells are administered. To further enhance angiogenesis an endothelial progenitor cell modified to express an endothelial cell mitogen may be used. Additionally, an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen can further be administered.

In another embodiment, the present invention provides methods of enhancing angiogenesis or treating an injured blood vessel. In accordance with these methods, endothelial progenitor cells are isolated from the patient, preferably from peripheral blood, and readministering to the patient. The patient may also be treated with endothelial cell mitogens to endothelial cell growth. The vessel injury can be the result of balloon angioplasty, deployment of an endovascular stent or a vascular graft.

The present invention also provides a method of screening for the presence of ischemic tissue or vascular injury in a patient. The method involves contacting the patient with a labeled EC progenitor and detecting the labeled cells at the site of the ischemic tissue or vascular injury.

The present invention also includes pharmaceutical products and kit for all the uses contemplated in the methods described herein.

Other aspects of the invention are disclosed infra.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1G show cell shape and formation. FIG. 1A shows spindle shaped attaching cells (AT^{CD34+}) 7 days after plating MB^{CD34+} on fibronectin with standard medium (10). Network formation (1B) and cord-like structures (1C) were observed 48 h after plating co-culture of MB^{CD34+}, labeled with Dil dye (Molecular Probe), and unlabeled MB^{CD34+} (ratio of 1:100) on fibronectin-coated dish. These cords consisted principally of Dil-labeled MB^{CD34+} derived cells (AT^{CD34+}). Beginning 12 h after co-culture, MB^{CD34+} derived cells demonstrated multiple foci of cluster formation (1D,1E.). AT^{CD34+} sprout from the periphery, while round cells remain in the center and detach from the cluster several days later. After 5 d, uptake of acLDL-Dil (Molecular Probe) was seen in AT^{CD34+} at the periphery but not the center of the cluster (1F,1G).

FIG. 2 shows the number of AT^{CD34+} 12 h and 3 d after single culture of MB^{CD34+} on plastic alone (CD34+/non), collagen coating (CD34+/COL), or fibronectin (CD34+/FN), and MB^{CD34+} on fibronectin (CD34-/FN). AT^{CD34+} yielded significantly higher number of cells at 12 h and 3 d when plated on fibronectin ($p < 0.05$, by ANOVA).

FIG. 3 shows FACS analysis of freshly isolated MB^{CD34+}, AT^{CD34+} after 7 days in culture, and HUVECs. Cells were labeled with FITC using antibodies against CD34, CD31 (Bioss); Flk-1, Tie-2 (Santa Cruz Biotechnology); and CD45. All results were confirmed by triplicate experiments. Shaded area of each box denotes negative antigen gate, white area denotes positive gate. Numbers indicated for individual gates denote percentage of cells determined by comparison with corresponding negative control labeling.

FIG. 4 shows expression of eNOS mRNA in MB^{CD34+}, MB^{CD34+}, AT^{CD34+}, human coronary smooth muscle cells (HCSMCs) and HUVECs. DNA was reverse transcribed from 1×10^6 cells each. Equal aliquots of the resulting DNA were amplified by PCR (40 cycles) with paired primers (sense/antisense: AAG ACA TTT TCG GGC TCA CGC TGC GCA CCC/TGG GGT AGG CAC TTT AGT AGT TCT

CCT AAC, SEQ ID NO:2) to detect eNOS mRNA. Equal aliquots of the amplified product were analyzed on a 1% agarose gel. Only a single band was observed, corresponding to the expected size (548 bp) for eNOS. Lane 1=MB^{CD34+}, Lane 2=MB^{CD34+}, Lane 3=AT^{CD34+} after 3 d, Lane 4=AT^{CD34+} after 7 d, Lane 5=HCSMCs, Lane 6=HUVECs.

FIG. 5 is a graph illustrating NO release from AT^{CD34+} was measured with an NO-specific polarographic electrode connected to an NO meter (Iso-NO, World Precision Instruments) (17). Calibration of NO electrode was performed daily before experimental protocol according to the following equation: $2\text{KNO}_3 + 2\text{KI} + 2\text{H}_2\text{SO}_4 \rightarrow 2\text{NO} + \text{I}_2 + 2\text{H}_2\text{O} + 2\text{K}_2\text{SO}_4$. standard calibration curve was obtained by adding graded concentrations of KNO₃ (0-500 nmol/L) to calibration solution containing KI and H₂SO₄. Specificity of the electrode to NO was previously documented by measurement of NO from authentic NO gas (18). AT^{CD34+} cultured in 6-well plate were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments) to maintain temperature between 35 and 37° C. For NO measurement, sensor probe was inserted vertically into the wells, and the tip of the electrode remained 2 mm under the surface of the solution. Measurement of NO, expressed as pmol/10⁵ cells, was performed in a well with incremental doses of VEGF (1, 10, 100 ng/ml) and Ach (0.1, 1, 10 μ M). HUVECs and bovine aortic ECs were employed as positive controls. For negative control, HCSMCs, NO was not detectable. All values reported represent means of 10 measurements for each group.

FIGS. 6A-6D show co-culture of MB^{CD34+} with HUVECs. Freshly isolated MB^{CD34+} were labeled with Dil dye and plated on a confluent HUVEC monolayer attached to a fibronectin-coated chamber slide at a density of 278 cells/mm² (Nunc). Differentiation of MB^{CD34+} into spindle shaped attaching cells (AT^{CD34+}) (red fluorescence) was observed among HUVECs within 12 h (6A). The AT^{CD34+} number increased on monolayer for 3 d (6B), while mesh-work structures were observed in some areas (6C). Three days after co-culture, both cells were re-seeded on Matrigel (Becton Dickinson)-coated slides and within 12 h disclosed capillary network formation, consisting of Dil-labeled AT^{CD34+} and HUVECs (6D).

FIG. 7 shows the effect of activated ECs and VEGF on MB^{CD34+} differentiation was investigated by pretreatment of HUVEC with TNF- α (20 ng/ml) for 12 h, and/or incubation of AT^{CD34+}/HUVEC co-culture with VEGF (50 ng/ml).

FIGS. 8A-8K show sections retrieved from ischemic hindlimb following in vivo administration of heterologous (FIGS. 8A-8H), homologous (8I), and autologous (8J,8K) EC progenitors. (8A,8B) Red fluorescence in small inter-muscular artery 6 wks after injection of Dil-labeled MB^{CD34+}. Green fluorescence denotes EC-specific lectin UEA-1. (8C) Dil (red) and CD31 (green) in capillaries between muscles, photographed through double filter 4 wks after Dil-labeled MB^{CD34+} injection. (8D) Same capillary structure as in (C), showing CD31 expression by MB^{CD34+}, which have been incorporated into host capillary structures expressing CD31. (8E,8F) Immunostaining 2 wks after MB^{CD34+} injection shows capillaries comprised of Dil-labeled MB^{CD34+} derived cells expressing tie-2 receptor (green fluorescence). Most MB^{CD34+} derived cells are tie-2 positive, and are integrated with some tie-2 positive native (host) capillary cells identified by absence of red fluorescence. (8G,8H) Two wks after injection of Dil-labeled MB^{CD34+}. Although isolated MB^{CD34+} derived cells (red) can be observed between muscles, but these cells do not express CD31.

(8I) Immunohistochemical, β -galactosidase staining of muscle harvested from ischemic limb of B6, 129 mice 4 wks following administration of MB^{Flk-1+} isolated from β -galactosidase transgenic mice. Cells overexpressing β -galactosidase (arrows) have been incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 antibody and BS-1 lectin.

(8J,8K) Sections of muscles harvested from rabbit ischemic hindlimb 4 wks after administration of autologous MB^{CD34+}. Dil fluorescence (J) indicates localization of MB^{CD34+} derived cells in capillaries seen in phase contrast photomicrograph (8K). Each scale bar indicates 50 μ m.

FIG. 9 is a photograph from a scanning electron microscope showing that EC progenitors had adhered to the denuded arterial surface and assumed a morphology suggestive of endothelial cells.

DETAILED DESCRIPTION OF THE INVENTION

We have now discovered a means to regulate angiogenesis, to promote angiogenesis in certain subject populations, and to more precisely target certain tissues. These methods all involve the use of endothelial cell progenitors. One preferred progenitor cell is an angioblast.

Post-natal neovascularization is believed to result exclusively from the proliferation, migration, and remodeling of fully differentiated endothelial cells (ECs) derived from pre-existing native blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to formation of embryonic blood vessels from EC progenitors (2).

In contrast to angiogenesis, vasculogenesis typically begins as a cluster formation, or blood island, comprised of EC progenitors (e.g. angioblasts) at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this intimate and predictable spatial association, such EC progenitors and HSCs share certain common antigenic determinants, including flk-1, tie-2, and CD-34. Consequently, these progenitor cells have been interpreted to derive from a common hypothetical precursor, the hemangioblast (3,4).

The demonstration that transplants of HSCs derived from peripheral blood can provide sustained hematopoietic recovery constitutes inferential evidence for circulating stem cells. (5). This observation is now being exploited clinically as an alternative to bone marrow transplantation.

We have now discovered that by using techniques similar to those employed for HSCs, EC progenitors can be isolated from circulating blood. In vitro, these cells differentiate into ECs. Indeed, one can use a multipotentiate undifferentiated cell as long as it is still capable of becoming an EC, if one adds appropriate agents to result in it differentiating into an EC.

We have also discovered that in vivo, heterologous, homologous, and autologous EC progenitor grafts incorporate into sites of active angiogenesis or blood vessel injury, i.e. they selectively migrate to such locations. This observation was surprising. Accordingly, one can target such sites by the present invention.

In accordance with the present invention, EC progenitors can be used in a method for regulating angiogenesis, i.e., enhancing or inhibiting blood vessel formation, in a selected patient and in some preferred embodiments for targetting specific locations. For example, the EC progenitors can be used to enhance angiogenesis or to deliver an angiogenesis

modulator, e.g. anti- or pro-angiogenic agents, respectively to sites of pathologic or utilitarian angiogenesis. Additionally, in another embodiment, EC progenitors can be used to induce reendothelialization of an injured blood vessel, and thus reduce restenosis by indirectly inhibiting smooth muscle cell proliferation.

In one preferred embodiment the EC cells can be used alone to potentiate a patient for angiogenesis. Some patient population, typically elderly patients, may have either a limited number of ECs or a limited number of functional ECs. Thus, if one desires to promote angiogenesis, for example, to stimulate vascularization by using a potent angiogenesis promotor such as VEGF, such vascularization can be limited by the lack of ECs. However, by administering the EC progenitors one can potentiate the vascularization in those patients.

Accordingly, the present method permits a wide range of strategies designed to modulate angiogenesis such as promoting neovascularization of ischemic tissues (24). EC mitogens such as VEGF and bFGF, for example, have been employed to stimulate native ECs to proliferate, migrate, remodel and thereby form new sprouts from parent vessels (25). A potentially limiting factor in such therapeutic paradigms is the resident population of ECs that is competent to respond to administered angiogenic cytokines. The finding that NO production declines as a function of age (26) may indicate a reduction in EC number and/or viability that could be addressed by autologous EC grafting. The success demonstrated to date with autologous grafts of HSCs derived from peripheral blood (5) supports the clinical feasibility of a "supply side" approach to therapeutic angiogenesis. The in vivo data set forth herein indicate that autologous EC transplants are feasible, and the in vitro experiments indicate that EC progenitors (MB^{CD34+} derived ECs) can be easily manipulated and expanded ex vivo.

Our discovery that these EC progenitors home to foci of angiogenesis makes these cells useful as autologous vectors for gene therapy and diagnosis of ischemia or vascular injury. For example, these cells can be utilized to inhibit as well as augment angiogenesis. For anti-neoplastic therapies, for example, EC progenitors can be transfected with or coupled to cytotoxic agents, cytokines or co-stimulatory molecules to stimulate an immune reaction, other anti-tumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by prior transfection of EC progenitors to achieve constitutive expression of angiogenic cytokines and/or selected matrix proteins (27). In addition, the EC progenitors may be labelled, e.g., radiolabelled, administered to a patient and used in the detection of ischemic tissue or vascular injury.

EC progenitors may be obtained from human mononuclear cells obtained from peripheral blood or bone marrow of the patient before treatment. EC progenitors may also be obtained from heterologous or autologous umbilical cord blood. Peripheral blood is preferred due to convenience. The leukocyte fraction of peripheral blood is most preferred. EC progenitors may be isolated using antibodies that recognize EC progenitor specific antigens on immature human hematopoietic progenitor cells (HSCs). For example, CD34 is commonly shared by EC progenitor and HSCs. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (6). It is also expressed by many, including most activated, ECs in the adult (7). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (8), is also expressed by both early HSCs and ECs, but ceases to be expressed in the course of hematopoietic differentiation (9).

To obtain the EC progenitors from peripheral blood about 5 ml to about 500 ml of blood is taken from the patient. Preferably, about 50 ml to about 200 ml of blood is taken.

EC progenitors can be expanded *in vivo* by administration of recruitment growth factors, e.g., GM-CSF and IL-3, to the patient prior to removing the progenitor cells.

Methods for obtaining and using hematopoietic progenitor cells in autologous transplantation are disclosed in U.S. Pat. No. 5,199,942, the disclosure of which is incorporated by reference.

Once the progenitor cells are obtained by a particular separation technique, they may be administered to a selected patient to treat a number of conditions including, for example, unregulated angiogenesis or blood vessel injury. The cells may also be stored in cryogenic conditions. Optionally, the cells may be expanded *ex vivo* using, for example, the method disclosed by U.S. Pat. No. 5,541,103, the disclosure of which is incorporated by reference.

The progenitor cells are administered to the patient by any suitable means, including, for example, intravenous infusion, bolus injection, and site directed delivery via a catheter. Preferably, the progenitor cells obtained from the patient are readministered. Generally, from about 10^6 to about 10^{10} progenitor cells are administered to the patient for transplantation.

Depending on the use of the progenitor cells, various genetic material may be delivered to the cell. The genetic material that is delivered to the EC progenitors may be genes, for example, those that encode a variety of proteins including anticancer agents. Such genes include those encoding various hormones, growth factors, enzymes, cytokines, receptors, MHC molecules and the like. The term "genes" includes nucleic acid sequences both exogenous and endogenous to cells into which a virus vector, for example, a pox virus such as swine pox containing the human TNF gene may be introduced. Additionally, it is of interest to use genes encoding polypeptides for secretion from the EC progenitors so as to provide for a systemic effect by the protein encoded by the gene. Specific genes of interest include those encoding TNF, TGF- α , TGF- β , hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin-12 etc., GM-CSF, G-CSF, M-CSF, human growth factor, co-stimulatory factor B7, insulin, factor VIII, factor IX, PDGF, EGF, NGF, IL-1ra, EPO, β -globin, EC mitogens and the like, as well as biologically active muteins of these proteins. The gene may further encode a product that regulates expression of another gene product or blocks one or more steps in a biological pathway. In addition, the gene may encode a toxin fused to a polypeptide, e.g., a receptor ligand, or an antibody that directs the toxin to a target, such as a tumor cell. Similarly, the gene may encode a therapeutic protein fused to a targeting polypeptide, to deliver a therapeutic effect to a diseased tissue or organ.

The cells can also be used to deliver genes to enhance the ability of the immune system to fight a particular disease or tumor. For example, the cells can be used to deliver one or more cytokines (e.g., IL-2) to boost the immune system and/or one or more antigens.

These cells may also be used to selectively administer drugs, such as an antiangiogenesis compound such as O-chloroacetyl carbamoyl fumagillol (TNP-470). Preferably the drug would be incorporated into the cell in a vehicle such as a liposome, a timed released capsule, etc. The EC progenitor would then selectively home in on a site of active angiogenesis such as a rapidly growing tumor where the compound would be released. By this method, one can reduce undesired side effects at other locations.

In one embodiment, the present invention may be used to enhance blood vessel formation in ischemic tissue, i.e., a tissue having a deficiency in blood as the result of an ischemic disease. Such tissues can include, for example, muscle, brain, kidney and lung. Ischemic diseases include, for example, cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

If it is desirable to further enhance angiogenesis, endothelial cell mitogens may also be administered to the patient in conjunction with, or subsequent to, the administration of the EC progenitor cells. Endothelial cell mitogens can be administered directly, e.g., intra-arterially, intramuscularly, or intravenously, or nucleic acid encoding the mitogen may be used. See, Baffour, et al., *supra* (bFGF); Pu, et al., *Circulation*, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., *supra* (bFGF); Ferrara, et al., *Biochem. Biophys. Res. Commun.*, 161:851-855 (1989) (VEGF); (Takeshita, et al., *Circulation*, 90:228-234 (1994)).

The nucleic acid encoding the EC mitogen can be administered to a blood vessel perfusing the ischemic tissue or to a site of vascular injury via a catheter, for example, a hydrogel catheter, as described by U.S. Ser. No. 08/675,523, the disclosure of which is herein incorporated by reference. The nucleic acid also can be delivered by injection directly into the ischemic tissue using the method described in U.S. Ser. No. 08/545,998.

As used herein the term "endothelial cell mitogen" means any protein, polypeptide, mutein or portion that is capable of, directly or indirectly, inducing endothelial cell growth. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF- α and TGF- β), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor (TNF- α), hepatocyte growth factor (HGF), insulin like growth factor (IGF), erythropoietin, colony stimulating factor (CSF), macrophage-CSF (M-CSF), granulocyte/macrophage CSF (GM-CSF) and nitric oxidesynthase (NOS). See, Klagsbrun, et al., *Annu. Rev. Physiol.*, 53:217-239 (1991); Folkman, et al., *J. Biol. Chem.*, 267:10931-10934 (1992) and Symes, et al., *Current Opinion in Lipidology*, 5:305-312 (1994). Muteins or fragments of a mitogen may be used as long as they induce or promote EC cell growth.

Preferably, the endothelial cell mitogen contains a secretory signal sequence that facilitates secretion of the protein. Proteins having native signal sequences, e.g., VEGF, are preferred. Proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., *Nature*, 362:844 (1993).

The nucleotide sequence of numerous endothelial cell mitogens, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g., PCR amplification. A DNA encoding VEGF is disclosed in U.S. Pat. No. 5,332,671, the disclosure of which is herein incorporated by reference.

In certain situations, it may be desirable to use nucleic acids encoding two or more different proteins in order to optimize the therapeutic outcome. For example, DNA encoding two proteins, e.g., VEGF and bFGF, can be used,

and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-arginine, fibronectin, urokinase, plasminogen activator and heparin.

The term "effective amount" means a sufficient amount of compound, e.g. nucleic acid delivered to produce an adequate level of the endothelial cell mitogen, i.e., levels capable of inducing endothelial cell growth and/or inducing angiogenesis. Thus, the important aspect is the level of mitogen expressed. Accordingly, one can use multiple transcripts or one can have the gene under the control of a promoter that will result in high levels of expression. In an alternative embodiment, the gene would be under the control of a factor that results in extremely high levels of expression, e.g., *lat* and the corresponding *tar* element.

The EC progenitors may also be modified *ex vivo* such that the cells inhibit angiogenesis. This can be accomplished, for example, by introducing DNA encoding angiogenesis inhibiting agents to the cells, using for example the gene transfer techniques mentioned herein. Angiogenesis inhibiting agents include, for example, proteins such as thrombospondin (Dameron et al., *Science* 265:1582-1584 (1994)), angiostatin (O'Reilly et al., *Cell* 79:315-328 (1994)), IFN- α (Folkman, J. *Nature Med.* 1:27-31 (1995)), transforming growth factor beta, tumor necrosis factor alpha, human platelet factor 4 (PF4); substances which suppress cell migration, such as proteinase inhibitors which inhibit proteases which may be necessary for penetration of the basement membrane, in particular, tissue inhibitors of metalloproteinase TIMP-1 and TIMP-2; and other proteins such as protamine which has demonstrated angiostatic properties, decoy receptors, drugs such as analogues of the angiostatin fumagillin, e.g., TNP-470 (Ingber et al., *Nature* 348:555-557 (1990)), antibodies or antisense nucleic acid against angiogenic cytokines such as VEGF. Alternatively, the cells may be coupled to such angiogenesis inhibiting agent.

If the angiogenesis is associated with neoplastic growth the EC progenitor cell may also be transfected with nucleic acid encoding, or coupled to, anti-tumor agents or agents that enhance the immune system. Such agents include, for example, TNF, cytokines such as interleukin (IL) (e.g., IL-2, IL-4, IL-10, IL-12), interferons (IFN) (e.g., IFN- γ) and co-stimulatory factor (e.g., B7). Preferably, one would use a multivalent vector to deliver, for example, both TNF and IL-2 simultaneously.

The nucleic acids are introduced into the EC progenitor by any method which will result in the uptake and expression of the nucleic acid by the cells. These can include vectors, liposomes, naked DNA, adjuvant-assisted DNA, catheters, gene gun, etc.

Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses and HIV-based viruses. One

preferred HIV-based viral vector comprises at least two vectors wherein the gag and pol genes are from an HIV genome and the env gene is from another virus. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox or avipox vectors, herpesvirus vectors such as a herpes simplex 1 virus (HSV) vector [Geller, A. I. et al., *J. Neurochem.* 64: 487 (1995); Lim, F., et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); Geller, A. I. et al., *Proc. Natl. Acad. Sci. U.S.A.* 90:7603 (1993); Geller, A. I. et al., *Proc. Natl. Acad. Sci. USA* 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., *Nat. Genet.* 3: 219 (1993); Yang, et al., *J. Virol.* 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplit, M. G., et al., *Nat. Genet.* 8:148 (1994)].

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, CaPO_4 precipitation, DEAE dextran, electroporation, protoplast fusion, lipofection, cell microinjection, viral vectors and use of the "gene gun".

To simplify the manipulation and handling of the nucleic acid encoding the protein, the nucleic acid is preferably inserted into a cassette where it is operably linked to a promoter. The promoter must be capable of driving expression of the protein in cells of the desired target tissue. The selection of appropriate promoters can readily be accomplished. Preferably, one would use a high expression promoter. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, et al., *Hum Gene Ther* 4:151 (1993)) and MMT promoters may also be used. Certain proteins can be expressed using their native promoter. Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression such as a *lat* gene and *tar* element. This cassette can then be inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an *E. coli* origin of replication. See, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the β -lactamase gene for ampicillin resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. The cassette can also be bound to a nucleic acid binding moiety in a synthetic delivery system, such as the system disclosed in WO 95/22618.

If desired, the preselected compound, e.g. a nucleic acid such as DNA may also be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, *BioTechniques*, 6:682 (1988). See also, Felgner and Holm, *Bethesda Res. Lab. Focus*, 11(2):21 (1989) and Maurer, R. A., *Bethesda Res. Lab. Focus*, 11(2):25 (1989).

Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., *Proc. Natl. Acad. Sci. USA*, 89:2581-2584

(1992); Stratford-Perricadot, et al., *J. Clin. Invest.*, 90:626-630 (1992); and Rosenfeld, et al., *Cell*, 68:143-155 (1992).

The effective dose of the nucleic acid will be a function of the particular expressed protein, the target tissue, the patient and his or her clinical condition. Effective amount of DNA are between about 1 and 4000 μ g, more preferably about 1000 and 2000, most preferably between about 2000 and 4000.

Alternatively, the EC progenitors may be used to inhibit angiogenesis and/or neoplastic growth by delivering to the site of angiogenesis a cytotoxic moiety coupled to the cell. The cytotoxic moiety may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal or plant origin, or an enzymatically active polypeptide chain or fragment ("A chain") of such a toxin. Enzymatically active toxins and fragments thereof are preferred and are exemplified by diphtheria toxin A fragment, non-binding active fragments of diphtheria toxin, exotoxin A (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alphasarcin, certain Aleurites fordii proteins, certain Dianthin proteins, Phytolacca americana proteins (PAP, PAPII and PAP-S), Momordica charantia inhibitor, curcumin, crocin, Saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin, Ricin A chain, *Pseudomonas aeruginosa* exotoxin A and PAP are preferred.

Conjugates of the EC progenitors and such cytotoxic moieties may be made using a variety of coupling agents. Examples of such reagents are N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters such as dimethyl adelpimide HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene.

The enzymatically active polypeptide of the toxins may be recombinantly produced. Recombinantly produced ricin toxin A chain (rRTA) may be produced in accordance with the methods disclosed in PCT W085/03508 published Aug. 15, 1985. Recombinantly produced diphtheria toxin A chain and non-binding active fragments thereof are also described in PCT W085/03508 published Aug. 15, 1985.

The methods of the present invention may be used to treat blood vessel injuries that result in denuding of the endothelial lining of the vessel wall. For example, primary angioplasty is becoming widely used for the treatment of acute myocardial infarction. In addition, endovascular stents are becoming widely used as an adjunct to balloon angioplasty. Stents are useful for rescuing a sub-optimal primary result as well as for diminishing restenosis. To date, however, the liability of the endovascular prosthesis has been its susceptibility to thrombotic occlusion in approximately 3% of patients with arteries 3.3 mm or larger. If patients undergo stent deployment in arteries smaller than this the incidence of sub-acute thrombosis is even higher. Sub-acute thrombosis is currently prevented only by the aggressive use of anticoagulation. The combination of vascular intervention and intense anticoagulation creates significant risks with regard to peripheral vascular trauma at the time of the stent/angioplasty procedure. Acceleration of reendothelialization by administration of EC progenitors to a patient undergoing, or subsequent to, angioplasty and/or stent deployment can stabilize an unstable plaque and prevent re-occlusion.

The method of the present invention may be used in conjunction with the method for the treatment of vascular injury disclosed in PCT/US96/15813.

In addition, the methods of the present invention may be used to accelerate the healing of graft tissue, e.g., vascular grafts.

The present invention also includes pharmaceutical products for all the uses contemplated in the methods described herein. For example, there is a pharmaceutical product, comprising nucleic acid encoding an endothelial cell mitogen and EC progenitors, in a physiologically acceptable administrable form.

The present invention further includes a kit for the in vivo systemic introduction of an EC progenitor and an endothelial cell mitogen or nucleic acid encoding the same into a patient. Such a kit includes a carrier solution, nucleic acid or mitogen, and a means of delivery, e.g., a catheter or syringe. The kit may also include instructions for the administration of the preparation.

All documents mentioned herein are incorporated by reference herein in their entirety.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Method and Materials

Human peripheral blood was obtained using a 20 gauge intravenous catheter, discarding the first 3 ml. Leukocyte fraction of blood was obtained by Ficoll density gradient centrifugation and plated on plastic tissue culture for 1 hr to avoid contamination by differentiated adhesive cells.

Fluorescent activated cell sorting (FACS) was carried out with $>1 \times 10^6$ CD34 positive and negative mononuclear blood cells (MB^{CD34+}, MB^{CD34-}). Cells were analyzed with Becton-Dickinson FACS sorter and the Lysis II analysis program using antibodies to CD34 (Bioscience).

M-199 medium with 20% FBS and bovine brain extract (Clonetics) was used as standard medium for all cell culture experiments.

C57BL/6Jx129/SV background male mice (Hylan), 3 mo old and 20-30 g, were used in these experiments (n=24). Animals were anesthetized with 160 mg/kg intraperitoneally of pentobarbital. The proximal end of one femoral artery and distal portion of the corresponding saphenous artery were ligated, following which the artery, as well as all side-branches, were dissected free and excised. (All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee.)

New Zealand White rabbits (3.8-4.2 kg, n=4, Pine Acre Rabbitry) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xylazine (2 mg/kg). After a longitudinal incision, the femoral artery was dissected free along its entire length; all branches of the femoral artery were also dissected free. After ligating the popliteal and saphenous arteries distally, the external iliac artery proximally and all femoral arterial branches, the femoral artery was completely excised (23).

Isolation and Analysis

CD34 positive mononuclear blood cells (MB^{CD34+}) were isolated from peripheral blood by CD34 antibody-coated magnetic beads (Dynal) as described above.

FACS analysis indicated that $15.9 \pm 3.3\%$ of selected cells versus $<0.1\%$ of the remaining cells expressed CD34. Depleted (MB^{CD34-}) cells were used as controls. Fik-1 antibody was used for magnetic bead selection of Fik-1 positive mononuclear blood cells (MB^{Fik1+}).

MB^{CD34+} and MB^{CD34-} were plated separately in standard medium on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of $1 \times 10^3/\text{mm}^2$, a limited number of MB^{CD34+} attached, and became spindle shaped and proliferated for 4 wks. A subset of MB^{CD34+} plated on fibronectin promptly attached and became spindle shaped within 3 days (FIG. 1A); the number of attaching cells (AT^{CD34+}) in culture increased with time (FIG. 2). Attached cells were observed only sporadically among cultures of MB^{CD34-}, including

To confirm that spindle-shaped cells were derived from CD34 positive cells, MB^{CD34+} were labeled with the fluorescent dye, Dil, and co-plated with unlabeled MB^{CD34-} on fibronectin at an overall density of $5 \times 10^3/\text{mm}^2$; ratio of the two cell types was identical to that of the original mononuclear cell population (1% MB^{CD34+}, 99% MB^{CD34-}). Seven days later, Dil-labeled cells derived from MB^{CD34+}, initially accounting for only 1% of blood cells, accounted for 60.3±4.7% of total attaching cells analyzed by FACS. Co-incubation with MB^{CD34-} increased proliferation to $>10 \times$ MB^{CD34+} plated alone at a cell density of $5 \times 10^3/\text{mm}^2$ cell (d $3 = 131.3 \pm 26.8$ vs $9.7 \pm 3.5/\text{mm}^2$). MB^{CD34+}/MB^{CD34-} co-cultures also enhanced MB^{CD34+} differentiation, including formation of cellular networks and tube-like structures on fibronectin-coated plates (FIGS. 1B,C). These structures consisted principally of Dil-labeled MB^{CD34+} derived cells (FIG. 1C). Moreover, within 12 h of co-culture, multiple cluster formations were observed (FIG. 1D), consisting principally of Dil-labeled MB^{CD34+} derived cells (FIG. 1E). These clusters were comprised of round cells centrally, and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which induced ECs and gave rise to vascular structures in vitro (3). AT^{CD34+} at the cluster periphery were shown to take up Dil-labeled acetylated LDL, characteristic of EC lineage (13), whereas the round cells comprising the center of cluster did not (FIGS. 1F,G); the latter detached from the cluster several days later. Similar findings were observed in the experiments using MB^{Flk1+}.

Expression of Leukocyte and EC Markers

To further evaluate progression of MB^{CD34+} to an EC-like phenotype, cells were assayed for expression of leukocyte and EC markers. Freshly isolated MB^{CD34+} versus AT^{CD34+} cultured at densities of 1×10^3 cell/ mm^2 for 7 days were incubated with fluorescent-labeled antibodies and analyzed by FACS (FIG. 3). Leukocyte common antigen, CD45, was identified on 94.1% of freshly isolated cells, but was essentially lost by 7 d in culture (FIG. 3). Augmented expression of UEA-1, CD34, CD31, Flk-1, Tie-2 and E-selectin—all denoting EC lineage (14)—was detected among AT^{CD34+} after 7 days in culture, compared to freshly isolated MB^{CD34+}. CD68 expression, suggesting monocyte/macrophage lineage, was limited to $6.0 \pm 2.4\%$ cells.

Expression of Factor VIII, UEA-1, CD31, eNOS, and E-selectin was also documented by immunohistochemistry for AT^{CD34+} after 7 days culture (data not shown). After 3, 7, and 14 days in culture, more than 80% AT^{CD34+} took up Dil-labeled acLDL (13).

ECs uniquely express endothelial constitutive nitric oxide synthase (eNOS). Accordingly, MB^{CD34+}, MB^{CD34-} and AT^{CD34+} were investigated for expression of eNOS by RT-PCR (15). eNOS mRNA was not detectable among MB^{CD34-} and was present at very low levels in freshly isolated MB^{CD34+} (FIG. 4). In AT^{CD34+} cultured for 7 d, however, eNOS mRNA was markedly increased (FIG. 5).

Functional evidence of eNOS protein in AT^{CD34+} was documented by measurement of nitric oxide in response to the EC-dependent agonist, acetylcholine (ACh), and the EC-specific mitogen, vascular endothelial growth factor (VEGF) (16) (FIG. 5); the latter parenthetically constitutes evidence for a functional Flk-1 receptor as well among AT^{CD34+}.

Cell-Cell Interaction

Cell-cell interaction is considered to play a decisive role in cell signaling, differentiation, and proliferation during hematopoiesis (19) and angiogenesis (20). To study the impact of MB^{CD34+} interaction with mature ECs on the differentiation of MB^{CD34+} into an EC-like phenotype, Dil-labeled MB^{CD34+} were plated on a confluent HUVEC monolayer. Adherent, labeled cells were found throughout the culture within 12 h (FIG. 6A), and increased in number for up to 3 d (FIG. 6B). When incubated with 50 ng/ml VEGF and 10 ng/ml bFGF, a meshwork of cord-like structures comprised of both Dil-labeled and unlabeled cells could be seen within 3 d after co-culture (FIG. 6C). Both cell types were then re-seeded on Matrigel (Becton Dickinson) coated slides and within 12 h demonstrated formation of capillary networks comprised of Dil-labeled MB^{CD34+} derived cells and HUVECs (FIG. 6D). To facilitate cell-cell interaction, HUVECs were pre-treated with TNF- α (21), resulting in increased numbers of AT^{CD34+} (FIG. 6E); synergistic augmentation was observed upon co-incubation with VEGF. Identically treated co-cultures of HUVECs and Dil-labeled MB^{CD34-} yielded desquamated labeled cells and/or no cords. Similar findings were observed when EC precursors were isolated using MB^{Flk1+}.

In Vivo Angiogenesis

Previous studies have established that ECs constitute the principal cell responsible for in vivo angiogenesis (1). To determine if MB^{CD34+} can contribute to angiogenesis in vivo, we employed two previously characterized animal models of hindlimb ischemia. For administration of human MB^{CD34+}, C57BL/6Jx129/SV background athymic nude mice were employed to avoid potential graft-versus host complications. Two days later, when the limb was severely ischemic, mice were injected with 5×10^5 Dil-labeled human MB^{CD34+} or MB^{CD34-} via the tail vein. Histologic sections of limbs examined 1, 2, 4, and 6 wks later for the presence of Dil labeled cells revealed numerous Dil-labeled cells in the neo-vascularized ischemic hindlimb. Labeled cells were more numerous in MB^{CD34+} versus MB^{CD34-} injected mice, and almost all labeled cells appeared to be integrated into capillary vessel walls (FIG. 8A,C,E,G).

No labeled cells were observed in the uninjured limbs of either MB^{CD34+} or MB^{CD34-} injected mice. Dil labeled cells were also consistently co-labeled with immunostains for UEA-1 lectin (FIG. 8B), CD31 (FIG. 8D), and Tie-2 (FIG. 8F). In contrast, in hindlimb sections from mice injected with MB^{CD34-}, labeled cells were typically found in stroma near capillaries, but did not form part of the vessel wall, and did not label with UEA-1 or anti-CD31 antibodies (FIG. 8G,H).

A transgenic mouse overexpressing β -galactosidase was then used to test the hypothesis that homologous grafts of EC progenitors could contribute to neovascularization in vivo. Flk-1 cell isolation was used for selection of EC progenitors due to lack of a suitable anti-mouse CD34 antibody. Approximately 1×10^4 MB^{Flk1+} were isolated from whole blood of 10 β -galactosidase transgenic mice with B6, 129 genetic background. MB^{Flk1+} or the same number of MB^{Flk1-} were injected into B6, 129 mice with hindlimb ischemia of 2 days duration. Immunostaining of ischemic

tissue for β -galactosidase, harvested 4 wks after injection, demonstrated incorporation of cells expressing β -galactosidase in capillaries and small arteries (FIG. 8I); these cells were identified as ECs by staining with anti-CD31 antibody and BS-1 lectin.

Finally, in vivo incorporation of autologous MB^{CD34+} into foci of neovascularization was tested in a rabbit model of unilateral hindlimb ischemia. MB^{CD34+} were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand white rabbits immediately prior to surgical induction of unilateral hindlimb ischemia. Immediately following completion of the operative procedure, freshly isolated autologous Dil-labeled MB^{CD34+} were re-injected into the ear vein of the same rabbit from which the blood had been initially obtained. Four wks after ischemia, histologic sections of the ischemic limbs were examined. Dil-labeled cells were localized exclusively to neovascular zones of the ischemic limb, incorporated into capillaries and consistently expressing CD31 and UEA-1 (FIG. 8J,K).

Consistent with the notion that HSCs and ECs are derived from a common precursor, our findings suggest that under appropriate conditions, a subpopulation of MB^{CD34+} or MB^{Fik-1+} can differentiate into ECs in vitro. Moreover, the in vivo results suggest that circulating MB^{CD34+} or MB^{Fik-1+} in the peripheral blood may constitute a contingency source of ECs for angiogenesis. Incorporation of in situ differentiating EC progenitors into the neovasculature of these adult species is consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2,3). The fact that these cells do not incorporate into mature blood vessels not undergoing angiogenesis suggests that injury, ischemia, and/or active angiogenesis are required to induce in situ differentiation of MB^{CD34+} to ECs.

EXAMPLE II

EC Progenitors Augment Reendothelialization

Following balloon injury, a denuded rat carotid artery was immediately excised and placed in culture in HUVEC medium, and Dil labeled CD34+ EC progenitor cells were seeded onto the artery. After 1 wk, the artery was washed with PBS to remove non-adherent cells. Consistent with the ability of CD34+ cells to differentiate into filtering cells, Dil labeled cells were found within the smooth muscle cell layer of the artery.

Scanning electron microscopy of the intimal surface, however, showed that Dil-labeled cells also had adhered to the denuded arterial surface, assuming a morphology suggestive of ECs (FIG. 9). Dil labeled cells also incorporated into the capillary-like sprouts at the bare ends of the excised arterial segment, suggesting that CD34+ cells may be capable of participating in angiogenesis as well.

To determine if exogenously administered CD34+ EC progenitor cells can contribute to reendothelialization of a denuded arterial surface in vivo, freshly isolated human CD34+ or CD34- cells were Dil labeled and seeded onto a denuded carotid artery of a nude rat. Following balloon denudation, 1.0×10^6 labeled cells in PBS was introduced into the denuded artery via a 22 G catheter, which remained in the artery for 30 min before the needle was withdrawn. The external carotid artery was then ligated, the common and internal carotid arterial ligatures removed, and the incision closed. The next day the rat was anesthetized and the vasculature perfusion fixed with Histo Choice (Amresco). The denuded arterial segment was excised and examined for the presence of adherent Dil labeled cells, which were identified in arteries seeded with CD34+ cells, but not CD34- cells.

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ciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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This invention has been described in detail including the preferred embodiments thereof. However, it will be appre-

What is claimed is:

1. A method for inducing the formation of new blood vessels in an ischemic tissue in a patient in need thereof, comprising:

administering to said patient host an effective amount of an isolated endothelial progenitor cell to induce new blood vessel formation in said ischemic tissue, wherein said endothelial progenitor cell are CD34⁺, flk-1⁺ or tie-2⁺.

2. The method of claim 1, further comprising the step of administering to the patient an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen.

3. The method of claim 2, wherein the endothelial cell mitogen is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β , platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α , hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte/macrophage CSF and nitric oxide synthase.

4. The method of claim 3, wherein the endothelial cell mitogen is vascular endothelial growth factor.

5. The method of claim 1, wherein said patient is in need of treatment for cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

6. A method of enhancing blood vessel formation in a patient in need thereof, comprising:

a. selecting the patient in need thereof;

b. isolating endothelial progenitor cells from the patient, wherein said endothelial progenitor cell are CD34⁺, flk-1⁺ or tie-2⁺; and

- c. readministering the endothelial progenitor cells to the patient.
- 7. A method for treating an injured blood vessel in a patient in need thereof, comprising:
 - a. selecting the patient in need thereof; and
 - b. isolating endothelial progenitor cells from the patient, wherein said endothelial progenitor cell are CD34⁺, flk-1⁺ or tie-2⁺; and
 - c. readministering the endothelial progenitor cells to the patient.
- 8. The method of claim 7, wherein the injury is the result of balloon angioplasty.
- 9. The method of claim 7, wherein the injury is the result of deployment of an endovascular stent.

10. The method of claim 7, further comprising the step of administering to the patient an endothelial cell mitogen or a nucleic acid encoding an endothelial cell mitogen.

- 11. The method of claim 10, wherein the endothelial cell mitogen is selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β , platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α , hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, granulocyte-macrophage CSF and nitric oxidesynthase.

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EVIDENCE APPENDIX

ITEM NO. 3

**Asahara et al. February 14, 1997 publication in Science
entitled, "Isolation of Putative Progenitor Endothelial Cells for
Angiogenesis," cited by Appellant as Reference ABA
in 8th Supplemental Information Disclosure Statement
filed September 24, 2008**

Isolation of Putative Progenitor Endothelial Cells for Angiogenesis

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Marcy Silver, Rien van der Zee, Tong Li,
Bernhard Witzentblacher, Gina Schatteman, Jeffrey M. Isner*

Putative endothelial cell (EC) progenitors or angioblasts were isolated from human peripheral blood by magnetic bead selection on the basis of cell surface antigen expression. In vitro, these cells differentiated into ECs. In animal models of ischemia, heterologous, homologous, and autologous EC progenitors incorporated into sites of active angiogenesis. These findings suggest that EC progenitors may be useful for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis) and for delivering anti- or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis.

Postnatal neovascularization is thought to result exclusively from the proliferation, migration, and remodeling of fully differentiated ECs derived from preexisting blood vessels (1). This adult paradigm, referred to as angiogenesis, contrasts with vasculogenesis, the term applied to the formation of embryonic blood vessels from EC progenitors, or angioblasts (2).

Vasculogenesis begins as a cluster formation, or blood island, comprising angioblasts at the periphery and hematopoietic stem cells (HSCs) at the center (3). In addition to this spatial association, angioblasts and HSCs share certain antigenic determinants, including Flk-1, Tie-2, and CD34. Conceivably, then, these progenitor cells may derive from a common precursor (3, 4).

The demonstration that HSCs from peripheral blood can provide sustained hematopoietic recovery is inferential evidence for circulating stem cells (5). Here, we have investigated the hypothesis that peripheral blood contains cells that can differentiate into ECs (6). We exploited two antigens that are shared by angioblasts and HSCs to isolate putative angioblasts from the leukocyte fraction of peripheral blood. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate (7). It is also expressed by many including most activated ECs in the adult (8). Flk-1, a receptor for vascular endothelial growth factor (VEGF) (9), is also expressed by both early HSCs and ECs but ceases to be expressed during hematopoietic differentiation (10, 11).

CD34-positive mononuclear blood cells (MB^{CD34+}) were isolated from human peripheral blood by means of magnetic beads

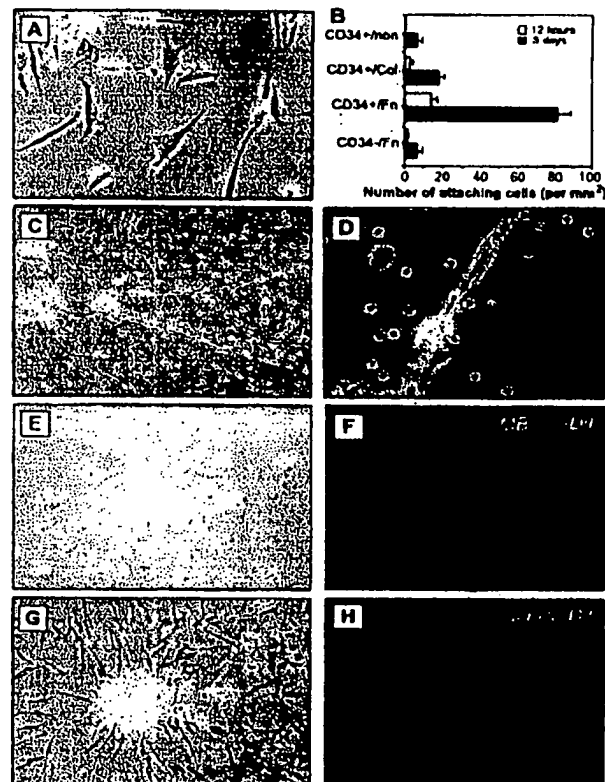
coated with antibody to CD34 (Dyna, Lake Success) (12). Fluorescence-activated cell sorting (FACS) analysis (13) indicated that $15.7 \pm 3.3\%$ of selected cells compared with $<0.1\%$ of the remaining cells expressed CD34. $CD34$ -depleted cells (MB^{CD34-}) were used as controls. An antibody to Flk-1 was used for magnetic bead selection of Flk-1-positive mononuclear

blood cells (MB^{Flk1+}); among MB^{Flk1+} cells, $20.0 \pm 3.3\%$ were Flk-1 positive.

The MB^{CD34+} and MB^{CD34-} cells were plated separately (14) on tissue culture plastic, collagen type I, or fibronectin. When plated on tissue culture plastic or collagen at a density of 1×10^4 cells/mm², a limited number of MB^{CD34+} attached, became spindle shaped, and proliferated for 4 weeks. A subset of MB^{CD34+} plated on fibronectin promptly attached and became spindle shaped within 3 days (Fig. 1A); the number of attaching cells (AT^{CD34+}) in culture increased with time (probability $P < 0.05$, by analysis of variance) (Fig. 1B). Attached cells were observed only sporadically among MB^{CD34-} cultures, including cells followed for up to 4 weeks on fibronectin-coated plates.

To confirm that the spindle-shaped cells were derived from $CD34$ -positive cells, we labeled MB^{CD34+} cells with the fluorescent dye Dil and coplanted them with unlabeled MB^{CD34-} cells on fibronectin at an overall density of 5×10^4 cells/mm²; the ratio of the two cell types was identical to that of the original mononuclear cell population ($1\% MB^{CD34+}$, $99\% MB^{CD34-}$). After 7 days, Dil-labeled cells derived from the MB^{CD34+} culture, which initially account-

Fig. 1. Attachment, cluster formation, and capillary network development by progenitor ECs in vitro. (A) Spindle-shaped attaching cells (AT^{CD34+}) 7 days after plating MB^{CD34+} (50 cells/mm²) on fibronectin in standard medium (14). (B) Number of AT^{CD34+} cells 12 hours and 3 days after culture of MB^{CD34+} on plastic alone ($CD34+/non$), collagen coating ($CD34+/Col$), or fibronectin ($CD34+/Fn$), and MB^{CD34-} on fibronectin ($CD34-/Fn$). Network formation (C) and cord-like structures (D) were observed 48 hours after plating coculture of MB^{CD34+} , labeled with Dil, with unlabeled MB^{CD34-} cells (ratio of 1:100) on fibronectin. At 12 hours after coculture, MB^{CD34+} -derived cells had formed multiple clusters (E and F). After 5 days, uptake of acLDL-Dil was detected in AT^{CD34+} cells at the periphery but not the center of the cluster (G and H).



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ed for only 1% of the blood cells, accounted for $60.3 \pm 4.7\%$ of total attaching cells as analyzed by FACS. Coincubation with MB^{CD34+} cells increased the proliferation rate to more than 10 times that of MB^{CD34+} plated alone. Cocultures of MB^{CD34+} and MB^{CD34+} cells also showed enhanced MB^{CD34+} differentiation, including the formation of cellular networks and tube-like structures on fibronectin-coated plates (Fig. 1, C and D). These structures consisted principally of Dil-labeled MB^{CD34+} -derived cells (Fig. 1D). Furthermore, within 12 hours of coculture, multiple clusters had formed (Fig. 1E) that contained mostly MB^{CD34+} -derived cells (Fig. 1F). These clusters comprised round cells centrally and sprouts of spindle-shaped cells at the periphery. The appearance and organization of these clusters resembled that of blood island-like cell clusters observed in dissociated quail epiblast culture, which gave rise to ECs and vascular structures in vitro (3). AT^{CD34+} cells at the cluster periphery took up Dil-labeled acetylated low density lipoprotein (acLDL), whereas the round cells did not (Fig. 1, G and H); the latter detached from the cluster several days later. The MB^{Flk1+} cells behaved similarly.

To evaluate whether MB^{CD34+} cells progressed to an EC-like phenotype, we assayed them for the expression of leukocyte and EC markers. Freshly isolated MB^{CD34+} cells, AT^{CD34+} cells cultured on fibronectin for 7 days, and human umbilical vein endothelial cells (HUVECs) were incubated with fluorescent-labeled antibodies and analyzed by FACS (Fig. 2). Leukocyte common antigen CD45 was identified on 94.1% of freshly

isolated cells but disappeared after 7 days of culture (Fig. 2). In freshly isolated MB^{CD34+} cells, $15.7 \pm 3.3\%$ were $CD34^+$, $27.6 \pm 4.3\%$ were $Flk-1^+$, and $10.8 \pm 0.9\%$ were $CD34^+Flk-1^+$. Expression of $CD34$, $CD31$, $Flk-1$, $Tie-2$, and E selectin—all markers of the EC lineage (11, 15)—was greater in AT^{CD34+} cells after 7 days of culture than in freshly isolated MB^{CD34+} cells.

Additional analyses (16) of AT^{CD34+} cells after 7 days of culture showed limited ($6.0 \pm 2.4\%$ cells) expression of CD68, a marker of the monocyte-macrophage lineage; positive immunostaining for factor VIII, ulex europaeus agglutinin-1 (UEA-1), CD31, endothelial constitutive nitric oxide synthase (ecNOS), and E selectin; and more than 80% uptake of Dil-labeled acLDL.

To confirm an EC-like phenotype of AT^{CD34+} cells, we documented expression of ecNOS, Flk-1/KDR (Flk-1 is also known as VEGFR-2 in mouse, and KDR is the human homolog of VEGFR-2), and CD31 mRNA at 7, 14, and 21 days by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3A). Evidence for ecNOS and Flk-1/KDR in AT^{CD34+} cells was also demonstrated in a functional assay. Nitric oxide was produced in the cells in response to the EC-dependent agonist acetylcholine (ACh) and the EC-specific mitogen VEGF (Fig. 3B); the latter response also confirms that the cells express a functional Flk-1 receptor (17).

To determine if MB^{CD34+} cells contribute to angiogenesis in vivo, we used mouse and rabbit models of hindlimb ischemia. For administration of human MB^{CD34+} cells, C57BL/6J \times 129/SV background athymic

nude mice were used to avoid potential graft-versus-host complications. Two days after creating unilateral hindlimb ischemia by excising one femoral artery, we injected mice with 5×10^5 Dil-labeled human MB^{CD34+} or MB^{CD34+} cells into the tail vein. Histologic examination 1 to 6 weeks later revealed numerous (Fig. 4A) including proliferative (Fig. 4, C and D) Dil-labeled cells in the neovascularized ischemic hindlimb. Nearly all labeled cells appeared integrated into capillary vessel walls. In MB^{CD34+} -injected mice, $13.4 \pm 5.7\%$ of all CD31-positive capillaries contained Dil-labeled cells, compared with $1.6 \pm 0.8\%$ in MB^{CD34+} -injected mice (18). By 6 weeks, Dil-labeled cells were clearly arranged into capillaries among preserved muscle structures (Fig. 4, I and J).

No labeled cells were observed in the uninjured limbs of either MB^{CD34+} or MB^{CD34+} -injected mice. Dil-labeled cells consistently colocalized with cells immunostained for CD31 (Fig. 4, B, F, and J), Tie-2 (Fig. 4G), and UEA-1 lectin (16). In contrast, in hindlimb sections from mice injected with MB^{CD34+} , Dil-labeled cells were typically found in stroma near capillaries, but they did not form part of the vessel wall nor did they colocalize with cells that stained with antibodies to either UEA-1 or CD31 (Fig. 4, K and L).

In a second set of mouse experiments, 1×10^4 MB^{Flk1+} cells were isolated from

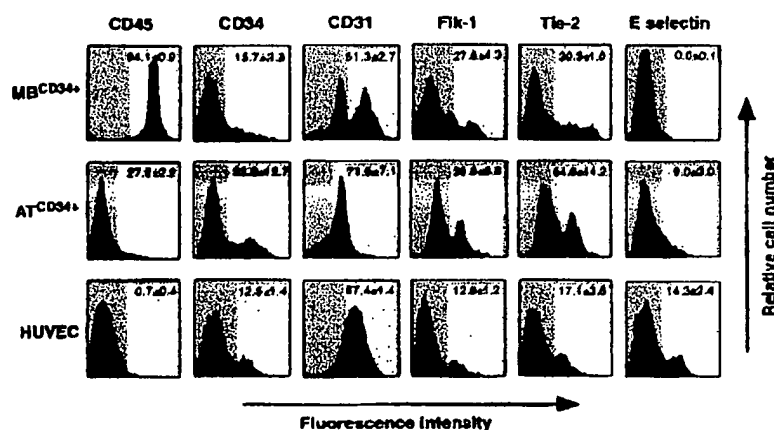


Fig. 2. FACS analysis of freshly isolated MB^{CD34+} and AT^{CD34+} cells after 7 days in culture, and HUVECs. Cells were labeled with fluorescent antibodies to CD45 (DAKO, Carpinteria); CD34, CD31 (Biossigen); Flk-1, Tie-2 (Santa Cruz); and E selectin (DAKO). Similar results were obtained in three or more experiments. The shaded area of each box denotes negative antigen gate, and the white area denotes positive gate. Numbers are the mean \pm SEM percentage of cells for all experiments determined by comparison with corresponding negative control labeling.

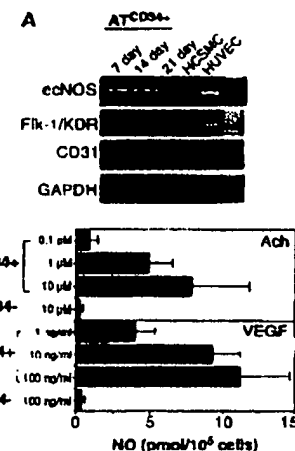


Fig. 3. Progenitor ECs express ecNOS, Flk-1/KDR, and CD31 mRNA and release NO. (A) Complementary DNA (from 10^6 cells) was amplified by PCR (40 cycles) with paired primers (23). (B) NO release from AT^{CD34+} and AT^{CD34-} cells cultured in six-well plates was measured as described (24). NO production was measured in a well with incremental doses of VEGF and ACh. HUVECs and hCSMCs were used as positive controls, and human coronary smooth muscle cells (hCSMCs) as negative control. The values are means \pm SEM of 10 measurements for each group.

whole blood of 10 transgenic mice constitutively overexpressing β -galactosidase (β -Gal) (all mice were $\text{Flk-1}^{+/+}$). $\text{MB}^{\text{Flk-1}+/+}$ or $\text{MB}^{\text{Flk-1}^{-/-}}$ cells were injected into nontransgenic mice of the same genetic background that had hindlimb ischemia of 2 days duration. Immunostaining of ischemic tissue, harvested 4 weeks after injection, for β -Gal demonstrated incorporation of cells expressing β -Gal in capillaries and small ar-

teries (Fig. 4M); these cells were identified as ECs by staining with antibody to CD31 (anti-CD31) and BS-1 lectin.

In vivo incorporation of autologous $\text{MB}^{\text{CD34}+/+}$ cells into foci of neovascularization was also tested in a rabbit model of unilateral hindlimb ischemia. $\text{MB}^{\text{CD34}+/+}$ cells were isolated from 20 ml of blood obtained by direct venipuncture of normal New Zealand White rabbits immediately

before surgical induction of unilateral hindlimb ischemia (19). Immediately after surgery, freshly isolated autologous Dil-labeled $\text{MB}^{\text{CD34}+/+}$ were reinjected into the ear vein of the same rabbit. Histologic examination of the ischemic limbs 4 weeks later revealed that Dil-labeled cells were localized exclusively to neovascular zones of the ischemic limb (Fig. 4, N and O) and were incorporated into $9.7 \pm 4.5\%$ of the capillaries that

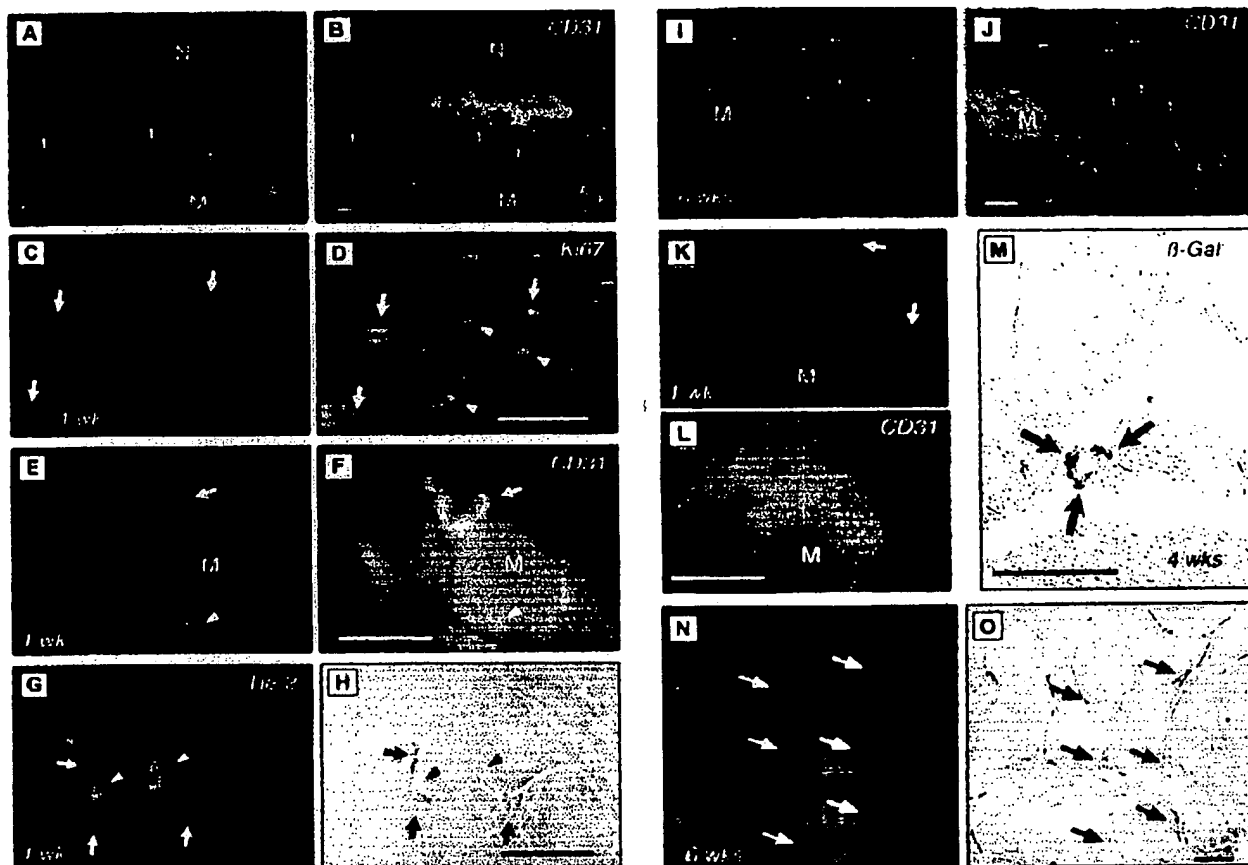


Fig. 4. Heterologous (panels A to L), homologous (M), or autologous (panels N and O) EC progenitors incorporate into sites of angiogenesis in vivo. (A and B) Dil-labeled $\text{MB}^{\text{CD34}+/+}$ (red, arrows) between skeletal myocytes (M), including necrotic (N) myocytes 1 week after injection; most are colabeled with CD31 (green, arrows). Note a preexisting artery (A), identified as CD31-positive, but Dil-negative. (C and D) Evidence of proliferative activity among several Dil-labeled $\text{MB}^{\text{CD34}+/+}$ -derived cells (red, arrows), indicated by coimmunostaining for antibody to K67 (Vector Lab, Burlingame, California) (green). Proliferative activity is also seen among Dil-negative, K67-positive capillary ECs (arrowheads); both cell types contribute to neovasculation. (E) Dil (red) and CD31 (green) in capillary ECs (arrows in E and F) between skeletal myocytes, photographed through a double filter 1 week after Dil-labeled $\text{MB}^{\text{CD34}+/+}$ injection. (F) A single green filter shows CD31 (green) expression in Dil-labeled capillary ECs integrated into the capillary with native (Dil-negative, CD31-positive) ECs (arrowheads in E and F). (G) Immunostaining 1 week after $\text{MB}^{\text{CD34}+/+}$ injection showing capillaries comprising Dil-labeled $\text{MB}^{\text{CD34}+/+}$ -derived cells expressing Tie-2 receptor (green). Several $\text{MB}^{\text{CD34}+/+}$ -derived cells (arrows) Tie-2 positive and integrated with some Tie-2-positive

host capillary cells (arrowheads) identified by the absence of red fluorescence. (H) Phase-contrast photomicrograph of the same section shown in (G) indicates the corresponding Dil-labeled (arrows) and -unlabeled (arrowheads) capillary ECs. (I and J) Six weeks after administration, $\text{MB}^{\text{CD34}+/+}$ -derived cells (red, arrows) colabel for CD31 in capillaries between preserved skeletal myocytes (M). (K and L) One week after injection of $\text{MB}^{\text{CD34}+/+}$, isolated $\text{MB}^{\text{CD34}+/+}$ -derived cells (red, arrows) are observed between myocytes but do not express CD31. (M) Immunostaining of β -Gal in a tissue section harvested from ischemic muscle of C57BL/6J,129/SV mice 4 weeks after the administration of $\text{MB}^{\text{Flk-1}+/+}$. Isolated $\text{MB}^{\text{Flk-1}+/+}$ cells overexpressing β -Gal (Flk-1 cell isolation was used for selection of EC progenitors because of the lack of a suitable antibody to mouse CD34.) Cells overexpressing β -Gal (arrows) were incorporated into capillaries and small arteries; these cells were identified as ECs by anti-CD31 and BS-1 lectin (16). (N and O) Section of muscle harvested from rabbit ischemic hindlimb 4 weeks after administration of autologous $\text{MB}^{\text{CD34}+/+}$ cells. Red fluorescence in (N) indicates localization of $\text{MB}^{\text{CD34}+/+}$ -derived cells in capillaries seen (arrows) in the phase-contrast photomicrograph in (O). Each scale bar is 50 μm .

consistently expressed CD31 and reacted with BS-1 lectin.

In summary, our findings suggest that cells isolated with anti-CD34 or anti-Flk-1 can differentiate into ECs in vitro. The in vivo results suggest that circulating MB^{CD34+} or MB^{Flk-1+} cells may contribute to neovascularization in adult species, consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis (2, 3). A potentially limiting factor in strategies designed to promote neovascularization of ischemic tissues (20) is the resident population of ECs that is competent to respond to administered angiogenic cytokines (21). This issue may be successfully addressed with autologous EC transplants. The fact that progenitor ECs home to foci of angiogenesis suggests potential utility as autologous vectors for gene therapy. For anti-neoplastic therapies, MB^{CD34+} cells could be transrected with or coupled to antitumor drugs or angiogenesis inhibitors. For treatment of regional ischemia, angiogenesis could be amplified by transfection of MB^{CD34+} cells to achieve constitutive expression of angiogenic cytokines or provisional matrix proteins or both (22).

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- Single donor human peripheral blood was obtained with a 20-gauge intravenous catheter. The first 3 ml was discarded, and the leukocyte fraction was obtained by Ficoll density gradient centrifugation. The cells were plated on plastic tissue culture for 1 hour to avoid contamination by differentiated adhesive cells.
- MB^{CD34+}, MB^{CD34-}, and MB^{Flk-1+} cells ($>1 \times 10^6$ of each) were analyzed with anti-CD34 (Bioss, Kennebunkport, ME) and anti-Flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA).
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- The mean percent of DiI-labeled capillaries among total CD31-positive capillaries was determined by averaging counts made in 10 randomly selected fields ($\times 400$).
- New Zealand White rabbits (3.8 to 4.2 kg, $n = 4$, Pine Acres Rabbits, Norton, MA) underwent ligation of the popliteal and saphenous arteries distally, the external iliac artery proximally, and all femoral arterial branches, after which the femoral artery was excised (S. Takeshita et al., *J. Clin. Invest.* **93**, 562 (1994); L. O. Fu et al., *Circulation* **88**, 208 (1993); R. Baffour et al., *J. Vasc. Med. Biol.* **16**, 181 (1992).
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- Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a positive control. The paired primers used (sense/antisense) were as follows: for cDNOS, AAG ACA TTT TCG GGC TCA CGC TGC GCA CCG/ TGG GGT ACC CAC TTT AGT AGT TCT CCT AAC (548-bp pairs (bp) PCR product); for Flk-1 (KDR), CAA CAA AGT CGG GAG AGG AGG/ATG ACG ATG GAC AAG TAG CC (819-bp PCR product); for CD31, GCT GT T GGT GGA AGG AGT GCA/GAA GTT GGC TGG AGG TGC TC (845-bp PCR product); for GAPDH, TGA AGG TCG GAG TCA ACG GAT TTG/ CAT GTG GGC CAT GAG GTC CAC GAC (983-bp PCR product).
- NO release was measured with a NO-specific polarographic electrode connected to a NO meter (iso-NO, World Precision Instruments, Sarasota, FL). AT^{CD34+} or AT^{CD34-} cells cultured in six-well plates were washed and then bathed in 5 ml of filtered Krebs-Henseleit solution. Cell plates were kept on a slide warmer (Lab Line Instruments, Melrose Park, IL) to maintain temperature between 35° and 37°C. The sensor probe was inserted vertically into the wells, and the tip of the electrode was positioned 2 mm under the surface of the solution.
- Supported by grants from NIH National Heart, Lung, and Blood Institute numbers 02824, 53354, and 57516, the American Heart Association, the E. L. Wiegand Foundation, and in part by the Uehara Memorial Foundation (T.M.).

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Somatic Frameshift Mutations in the BAX Gene in Colon Cancers of the Microsatellite Mutator Phenotype

Nicholas Rampino, Hiroyuki Yamamoto, Yuriy Ionov, Yan Li, Hisako Sawai, John C. Reed, Manuel Perucho*

Cancers of the microsatellite mutator phenotype (MMP) show exaggerated genomic instability at simple repeat sequences. More than 50 percent (21 out of 41) of human MMP⁺ colon adenocarcinomas examined were found to have frameshift mutations in a tract of eight deoxyguanosines [(G)₈] within BAX, a gene that promotes apoptosis. These mutations were absent in MMP⁻ tumors and were significantly less frequent in (G)₈ repeats from other genes. Frameshift mutations were present in both BAX alleles in some MMP⁺ colon tumor cell lines and in primary tumors. These results suggest that inactivating BAX mutations are selected for during the progression of colorectal MMP⁺ tumors and that the wild-type BAX gene plays a suppressor role in a p53-independent pathway for colorectal carcinogenesis.

The MMP pathway for colon cancer is characterized by genomic instability that leads to the accumulation of deletion and insertion mutations at simple repeat sequences (1-3). The fixation of these slip-page-induced replication errors as mutations (4) is associated with defects in DNA mismatch repair (5). Colorectal MMP⁺ tumors frequently contain frameshift mutations in the type II transforming growth factor- β (TGF- β) receptor gene (6) but are usually wild type for the p53 tumor suppressor gene (1, 7). In addition to its central role in cell growth arrest (8), p53 also plays a role in apoptosis in response to DNA

damage (9). The p53 protein transactivates BAX (10), a member of the BCL2 gene family (11) that promotes apoptosis (12).

The human BAX gene contains a tract of eight consecutive deoxyguanosines in the third coding exon, spanning codons 38 to 41 (ATG GGG GGG GAG) (12). To determine whether this sequence is a mutational target in MMP⁺ tumor cells, we amplified by the polymerase chain reaction (PCR) the region containing the (G)₈ tract from various MMP⁺ tumor cell lines. This analysis revealed band shifts suggestive of insertions and deletions of one nucleotide in some of these tumor cells (Fig. 1A). Prostate (DU145) and colon (LS180) tumor cells exhibited PCR patterns indistinguishable from those amplified from plasmids containing a BAX fragment with mutant (G)₇ and (G)₉ tracts (Fig. 1A, P9 and P7).

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EVIDENCE APPENDIX

ITEM NO. 4

Nabel et al. U.S. Patent No. 5,328,470



US005328470A

United States Patent [19].

[11] Patent Number: 5,328,470

Nabel et al.

[45] Date of Patent: Jul. 12, 1994

[54] TREATMENT OF DISEASES BY
SITE-SPECIFIC INSTILLATION OF CELLS
OR SITE-SPECIFIC TRANSFORMATION OF
CELLS AND KITS THEREFOR

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both of Ann Arbor, Mich.

[73] Assignee: The Regents of the University of
Michigan, Ann Arbor, Mich.

[21] Appl. No.: 741,244

[22] Filed: Jul. 26, 1991

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 724,509, Jun. 28, 1991,
which is a continuation-in-part of Ser. No. 331,336,
Mar. 31, 1989, abandoned.

[51] Int. Cl.³ A61M 29/00

[52] U.S. Cl. 604/101; 604/96;
606/194

[58] Field of Search 604/52, 53, 96, 97,
604/101, 181, 269, 280; 424/424, 425, 93 B;
514/120; 606/192, 174; 128/656, 658

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Primary Examiner—C. Fred Rosenbaum

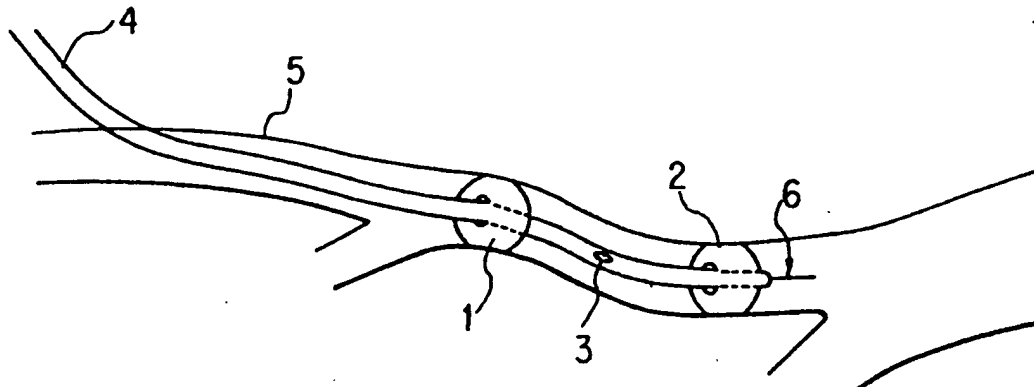
Assistant Examiner—V. Alexander

Attorney, Agent, or Firm—Oblon, Spivak, McClelland,
Maier & Neustadt

[57] ABSTRACT

A method for the direct treatment towards the specific
sites of a disease is disclosed. This method is based on
the delivery of proteins by catheterization to discrete
blood vessel segments using genetically modified or
normal cells or other vector systems. Endothelial cells
expressing recombinant therapeutic agent or diagnostic
proteins are situated on the walls of the blood vessel or
in the tissue perfused by the vessel in a patient. This
technique, provides for the transfer of cells or vectors
and expression of recombinant genes in vivo and allows
the introduction of proteins of therapeutic or diagnostic
value for the treatment of diseases.

10 Claims, 4 Drawing Sheets



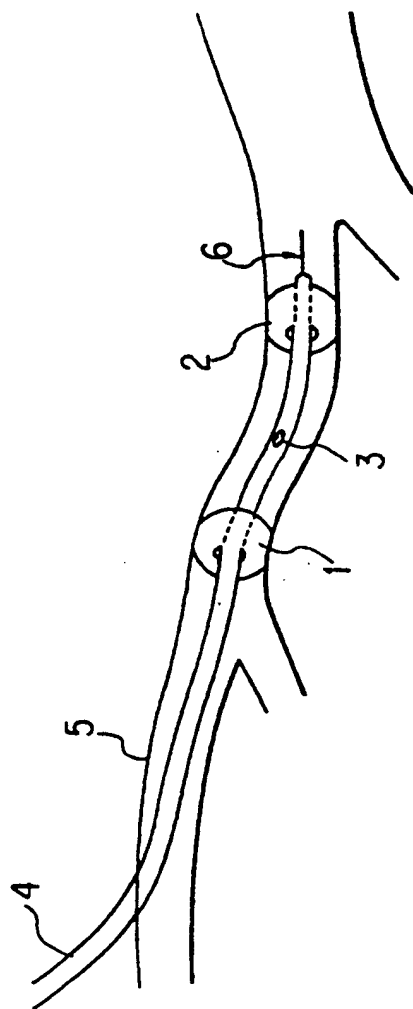


FIG. 1

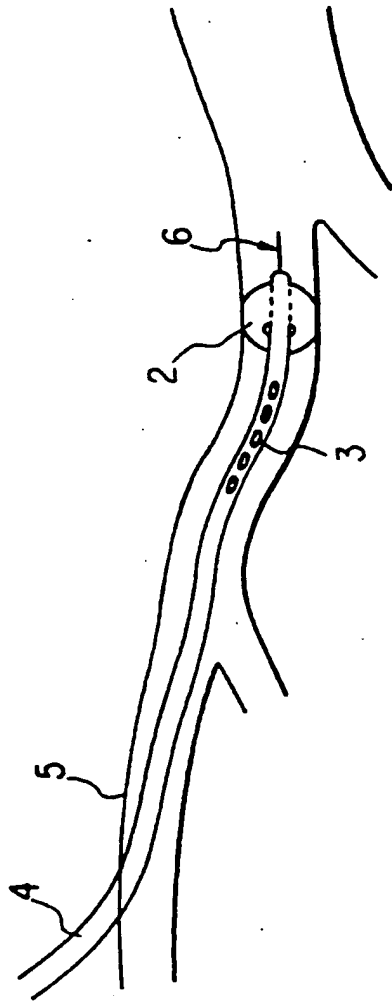
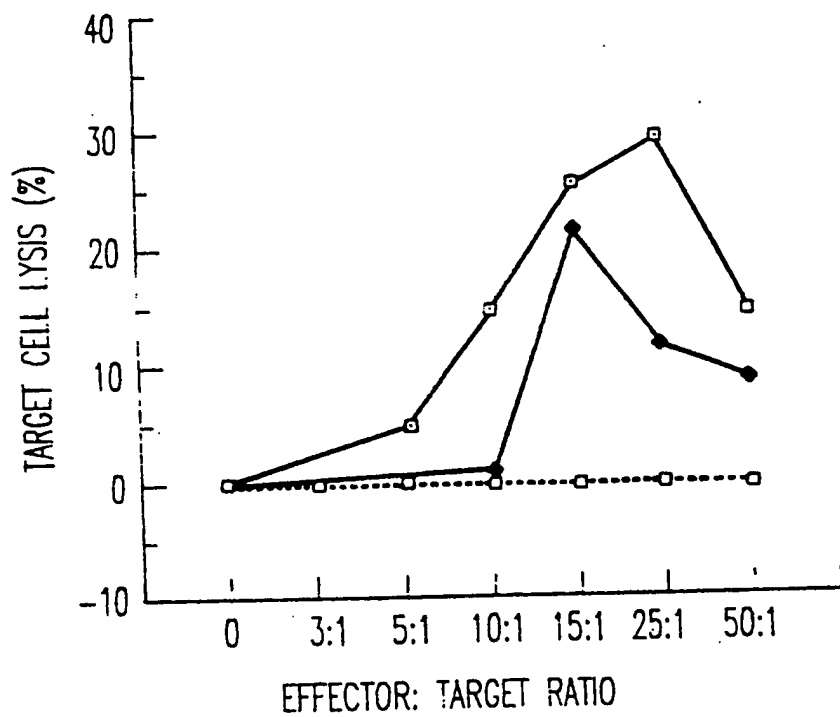


FIG. 2

FIG. 3I.V. INJECTIONS

□ — SOLUTION C/H-2Ks

◆ — LIPOFECTIN / H-2Ks

□ - - - LIPOFECTIN / RSV-B-GAL

WESTERN BLOT ANALYSIS

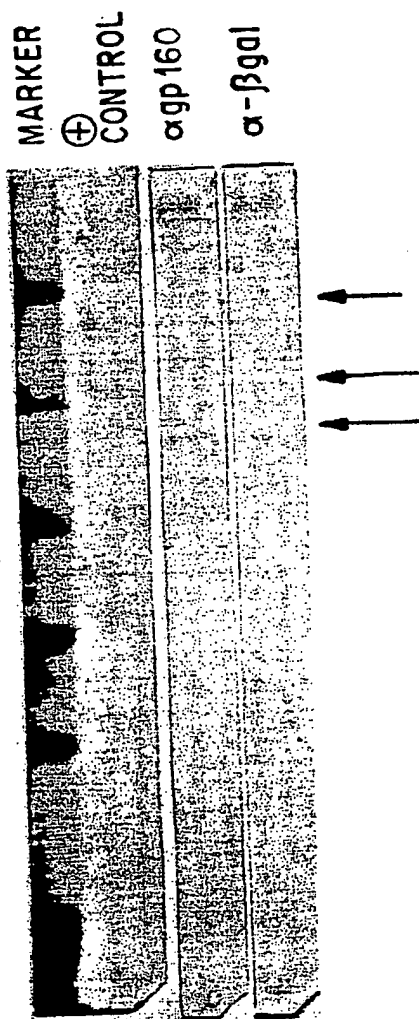


FIG. 4

TREATMENT OF DISEASES BY SITE-SPECIFIC INSTILLATION OF CELLS OR SITE-SPECIFIC TRANSFORMATION OF CELLS AND KITS THEREFOR

This is a continuation-in-part of U.S. patent application Ser. No. 07/724,509, filed on Jun. 28, 1991, now pending, which is a continuation-in-part of U.S. patent application Ser. No. 07/331,336, filed on Mar. 31, 1989, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the treatment of diseases by the site-specific instillation or transformation of cells and kits therefor. The present invention also relates to a method for modulating the immune system of an animal by the in vivo introduction of recombinant genes.

2. Discussion of the Background

The effective treatment of many acquired and inherited diseases remains a major challenge to modern medicine. The ability to deliver therapeutic agents to specific sites in vivo would be an asset in the treatment of, e.g., localized diseases. In addition the ability to cause a therapeutic agent to perfuse through the circulatory system would be effective for the treatment of, e.g., inherited diseases and acquired diseases or cancers.

For example, it would be desirable to administer in a steady fashion an antitumor agent or toxin in close proximity to a tumor. Similarly, it would be desirable to cause a perfusion of, e.g., insulin in the blood of a person suffering from diabetes. However, for many therapeutic agents there is no satisfactory method of either site-specific or systemic administration.

In addition, for many diseases, it would be desirable to cause, either locally or systemically, the expression of a defective endogenous gene, the expression of an exogenous gene, or the suppression of an endogenous gene. Again, these remain unrealized goals.

In particular, the pathogenesis of atherosclerosis is characterized by three fundamental biological processes. These are: 1) proliferation of intimal smooth muscle cells together with accumulated macrophages; 2) formation by the proliferated smooth muscle cells of large amounts of connective tissue matrix; and 3) accumulation of lipid, principally in the form of cholesterol esters and free cholesterol, within cells as well as in surrounding connective tissue.

Endothelial cell injury is an initiating event and is manifested by interference with the permeability barrier of the endothelium, alterations in the nonthrombogenic properties of the endothelial surface, and promotion of procoagulant properties of the endothelium. Monocytes migrate between endothelial cells, become active as scavenger cells, and differentiate into macrophages.

Macrophages then synthesize and secrete growth factors including platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor alpha (TGF- α). These growth factors are extremely potent in stimulating the migration and proliferation of fibroblasts and smooth muscle cells in the atherosclerotic plaque. In addition, platelets may interact with the injured endothelial cell and the activated macrophage to potentiate the elaboration of growth factors and thrombus formation.

Two major problems in the clinical management of coronary artery disease include thrombus formation in acute myocardial ischemia and restenosis following coronary angioplasty (PTCA). Both involve common cellular events, including endothelial injury and release of potent growth factors by activated macrophages and platelets. Coronary angioplasty produces fracturing of the atherosclerotic plaque and removal of the endothelium. This vascular trauma promotes platelet aggregation and thrombus formation at the PTCA site. Further release of mitogens from platelets and macrophages, smooth muscle cell proliferation and monocyte infiltration result in restenosis.

Empiric therapy with antiplatelet drugs has not prevented this problem, which occurs in one-third of patients undergoing PTCA. A solution to restenosis is to prevent platelet aggregation, thrombus formation, and smooth muscle cell proliferation.

Thrombus formation is also a critical cellular event in the transition from stable to unstable coronary syndromes. The pathogenesis most likely involves acute endothelial cell injury and/or plaque rupture, promoting dysjunction of endothelial cell attachment, and leading to the exposure of underlying macrophage foam cells. This permits the opportunity for circulating platelets to adhere, aggregate, and form thrombi.

The intravenous administration of thrombolytic agents, such as tissue plasminogen activator (tPA) results in lysis of thrombus in approximately 70% of patients experiencing an acute myocardial infarction. Nonetheless, approximately 30% of patients fail to reperfuse, and of those patients who undergo initial reperfusion of the infarct related artery, approximately 25% experience recurrent thrombosis within 24 hours. Therefore, an effective therapy for rethrombosis remains a major therapeutic challenge facing the medical community today.

As noted above, an effective therapy for rethrombosis is by far not the only major therapeutic challenge existing today. Others include the treatment of other ischemic conditions, including unstable angina, myocardial infarction or chronic tissue ischemia, or even the treatment of acquired and inherited diseases or cancers. These might be treated by the effective administration of anticoagulants, vasodilatory, angiogenic, growth factors or growth inhibitors to a patient. Thus, there remains a strongly felt need for an effective therapy in all of these clinical settings.

In addition, it is desirable to be able to modulate the immune system of an animal. In particular, much effort has been directed toward the development of vaccines to provide immunological protection from infection. However, the development of safe vaccines which can be readily administered to large numbers of patients is problematic, and for many diseases, such as, e.g., AIDS, no safe and effective vaccine is as yet available. Further, it is also sometimes desirable to specifically suppress an animal's immune response to prevent rejection of a transplant. Efforts to suppress transplant rejection have resulted in the development of drugs which result in a general suppression of the immune response, rather than specific suppression to transplantation antigens, and such drugs are not always effective. Thus, there remains a need for a method to modulate the immune system of an animal.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a novel method for the site-specific administration of a therapeutic agent.

It is another object of the present invention to provide a method for the perfusion of a therapeutic agent in the blood stream of a patient.

It is another object of the present invention to provide a method for causing the expression of an exogenous gene in a patient.

It is another object of the present invention to provide a method for causing the expression of a defective endogenous gene in a patient.

It is another object of the present invention to provide a method for suppressing the expression of an endogenous gene in a patient.

It is another object of the present invention to provide a method for site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the site-specific administration of a therapeutic agent or the perfusion of a therapeutic agent in the bloodstream of a patient.

It is another object of the present invention to provide a method for the treatment of a disease by causing either the expression of an exogenous gene, the expression of a defective endogenous gene, or the suppression of the expression of an endogenous gene in a patient.

It is another object of the present invention to provide a method for the treatment of a disease by site-specifically replacing damaged cells in a patient.

It is another object of the present invention to provide a kit for site-specifically instilling normal or transformed cells in a patient.

It is another object of the present invention to provide a kit for site-specifically transforming cells in vivo.

It is another object of the present invention to provide a method for modulating the immune system of an animal.

It is another object of the present invention to provide a method for modulating the immune system of an animal to sensitize the animal to a foreign molecule.

It is another object of the present invention to provide a method to stimulate the immune system of an animal to reject proteins in order to protect against infection by a microorganism or virus.

It is another object of the present invention to provide a method for modulating the immune system of an animal to tolerize the animal to a foreign molecule.

It is another object of the present invention to provide a method for modulating the immune system of an animal to reduce the tendency to reject a transplant.

It is another object of the present invention to provide a novel kit for transforming cells by systemic administration in vivo.

These and other objects of this invention which will become apparent during the course of the following detailed description of the invention have been discovered by the inventors to be achieved by (a) a method which comprises either (i) site-specific instillation of either normal (untransformed) or transformed cells in a patient or (ii) site-specific transformation of cells in a patient and (b) a kit which contains a catheter for (i) site-specific instillation of either normal or transformed cells or (ii) site-specific transformation of cells.

Site-specific instillation of normal cells can be used to replace damaged cells, while instillation of transformed cells can be used to cause the expression of either a defective endogenous gene or an exogenous gene or the suppression of an endogenous gene product. Instillation of cells in the walls of the patient's blood vessels can be used to cause the steady perfusion of a therapeutic agent in the blood stream.

The inventors have also discovered that by transforming cells of an animal, in vivo, it is possible to modulate the animal's immune system. In particular, by transforming cells of an animal, with a recombinant gene, by site-specific or systemic administration it is possible to modulate the animal's immune system to sensitize the animal to the molecule for which the recombinant gene encodes. Alternatively, by transforming cells of an animal with a recombinant gene, specifically at a site which determines the specificity of the immune system, such as, e.g., the thymus, it is possible to modulate the immune system of an animal to suppress the immune response to the molecule encoded by the recombinant gene.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying figures, wherein:

FIGS. 1 and 2 illustrate the use of a catheter in accordance with the invention to surgically or percutaneously implant cells in a blood vessel or to transform in vivo cells present on the wall of a patient's blood vessel;

FIG. 3 illustrates the relationship between the % of target cell lysis and the effector:target ratio for CTL cells; and

FIG. 4 illustrates the results of a Western blot analysis.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Thus, in one embodiment, the present invention is used to treat diseases, such as inherited diseases, systemic diseases, diseases of the cardiovascular system, diseases of particular organs, or tumors by instilling normal or transformed cells or by transforming cells.

The cells which may be instilled in the present method include endothelium, smooth muscle, fibroblasts, monocytes, macrophages, and parenchymal cells. These cells may produce proteins which may have a therapeutic or diagnostic effect and which may be naturally occurring or arise from recombinant genetic material.

Referring now to the figures, wherein like reference numerals designate identical or corresponding parts throughout the several views, and more particularly to FIG. 1 thereof, this figure illustrates the practice of the present invention with a catheter having a design as disclosed in U.S. Pat. No. 4,636,195, which is hereby incorporated by reference. This catheter may be used to provide normal or genetically altered cells on the walls of a vessel or to introduce vectors for the local transformation of cells. In the figure, 5 is the wall of the blood vessel. The figure shows the catheter body 4 held in place by the inflation of inflatable balloon means 1 and 2. The section of the catheter body 4 situated between balloon means 1 and 2 is equipped with instillation port.

means 3. The catheter may be further equipped with a guidewire means 6. FIG. 2 illustrates the use of a similar catheter, distinguished from the catheter illustrated in FIG. 1 by the fact that it is equipped with only a single inflatable balloon means 2 and a plurality of instillation port means 3. This catheter may contain up to twelve individual instillation port means 3, with five being illustrated.

In the case of delivery to an organ, the catheter may be introduced into the major artery supplying the tissue. Cells containing recombinant genes or vectors can be introduced through a central instillation port after temporary occlusion of the arterial circulation. In this way, cells or vector DNA may be delivered to a large amount of parenchymal tissue distributed through the capillary circulation. Recombinant genes can also be introduced into the vasculature using the double balloon catheter technique in the arterial circulation proximal to the target organ. In this way, the recombinant genes may be secreted directly into the circulation which perfuse the involved tissue or may be synthesized directly within the organ.

In one embodiment, the therapeutic agents are secreted by vascular cells supplying specific organs affected by the disease. For example, ischemic cardiomyopathy may be treated by introducing angiogenic factors into the coronary circulation. This approach may also be used for peripheral, vascular or cerebrovascular diseases where angiogenic factors may improve circulation to the brain or other tissues. Diabetes mellitus may be treated by introduction of glucose-responsive insulin secreting cells in the portal circulation where the liver normally sees a higher insulin concentration than other tissues.

In addition to providing local concentrations of therapeutic agents, the present method may also be used for delivery of recombinant genes to parenchymal tissues, because high concentrations of viral vector and other vectors can be delivered to a specific circulation. Using this approach, deficiencies of organ-specific proteins may also be treated. For example, in the liver, α -antitrypsin inhibitor deficiency or hypercholesterolemia may be treated by introduction of α -antitrypsin or the LDL receptor gene. In addition, this approach may be used for the treatment of a malignancy. Secretion of specific recombinant toxin genes into the circulation of inoperable tumors provides a therapeutic effect. Examples include acoustic neuromas or certain hemangiomas which are otherwise unresectable.

In clinical settings, these therapeutic recombinant genes are introduced in cells supplying the circulation of the involved organ. Although the arterial and capillary circulations are the preferred locations for introduction of these cells, venous systems are also suitable.

In its application to the treatment of local vascular damage the present invention provides for the expression of proteins which ameliorate this condition in situ. In one embodiment, because vascular cells are found at these sites, they are used as carriers to convey the therapeutic agents.

The invention thus, in one of its aspects, relies on genetic alteration of endothelial and other vascular cells or somatic cell gene therapy, for transmitting therapeutic agents (i.e., proteins, growth factors) to the localized region of vessel injury. To successfully use gene transplantation in the cells, four requirements must be fulfilled. First, the gene which is to be implanted into the cell must be identified and isolated. Second, the gene to

be expressed must be cloned and available for genetic manipulation. Third, the gene must be introduced into the cell in a form that will be expressed or functional. Fourth, the genetically altered cells must be situated in the vascular region where it is needed.

In accordance with the present invention the altered cells or appropriate vector may be surgically, percutaneously, or intravenously introduced and attached to a section of a patient's vessel wall. Alternatively, some of the cells existing on the patient's vessel wall are transformed with the desired genetic material or by directly applying the vector. In some instances, vascular cells which are not genetically modified can be introduced by these methods to replace cells lost or damaged on the vessel surface.

Any blood vessel may be treated in accordance with this invention; that is, arteries, veins, and capillaries. These blood vessels may be in or near any organ in the human, or mammalian, body.

Introduction of normal or genetically altered cells into a blood vessel

This embodiment of the invention may be illustrated as follows:

I. Establishment of endothelial or other vascular cells in tissue culture.

Initially, a cell line is established and stored in liquid nitrogen. Prior to cryopreservation, an aliquot is taken for infection or transfection with a vector, viral or otherwise, containing the desired genetic material.

Endothelial or other vascular cells may be derived enzymatically from a segment of a blood vessel, using techniques previously described in J. W. Ford, et al., *In Vitro*, 17, 40 (1981). The vessel is excised, inverted over a stainless steel rod and incubated in 0.1% trypsin in Ca^{++} - and Mg^{++} -free Hank's balanced salt solution (BSS) with 0.125% EDTA at pH 8 for 10 min at 37° C.

Cells (0.4 to 1.5×10^6) are collected by centrifugation and resuspended in medium 199 (GIBCO) containing 10% fetal bovine serum, endothelial cell growth supplement (ECGS, Collaborative Research, Waltham, Mass.) at 25 $\mu\text{g}/\text{ml}$, heparin at 15 U/ml, and gentamicin (50 $\mu\text{g}/\text{ml}$). Cells are added to a 75 cm^2 tissue culture flask precoated with gelatin (2 mg/ml in distilled water). Cells are fed every second day in the above medium until they reach confluence.

After two weeks in culture, the ECGS and heparin may be omitted from the medium when culturing porcine endothelium. If vascular smooth muscle cells or fibroblasts are desired the heparin and ECGS can be omitted entirely from the culturing procedure. Aliquots of cells are stored in liquid nitrogen by resuspending to approximately 10^6 cells in 0.5 ml of ice cold fetal calf serum on ice. An equal volume of ice cold fetal calf serum containing 10% DMSO is added, and cells are transferred to a prechilled screw cap Corning freezing tube. These cells are transferred to a -70°C freezer for 3 hours before long term storage in liquid nitrogen.

The cells are then infected with a vector containing the desired genetic material.

II. Introduction of cells expressing normal or exogenous proteins into the vasculature.

A. Introduction of cells expressing relevant proteins by catheterization.

The patient is prepared for catheterization either by surgery or percutaneously, observing strict adherence to sterile techniques. A cutdown procedure is performed over the target blood vessel or a needle is in-

serted into the target blood vessel after appropriate anesthesia. The vessel (5) is punctured and a catheter, such as described in U.S. Pat. No. 4,636,195, which is hereby incorporated by reference (available from USC1, Billerica, Mass.) is advanced by guidewire means (6) under fluoroscopic guidance, if necessary, into the vessel (5) (FIG. 1). This catheter means (4) is designed to introduce infected endothelial cells into a discrete region of the artery. The catheter has a proximal and distal balloon means (2) and (1), respectively, (e.g., each balloon means may be about 3 mm in length and about 4 mm in width), with a length of catheter means between the balloons. The length of catheter means between the balloons has a port means connected to an instillation port means (3). When the proximal and distal balloons are inflated, a central space is created in the vessel, allowing for instillation of infected cells through the port.

A region of the blood vessel is identified by anatomical landmarks and the proximal balloon means (2) is inflated to denude the endothelium by mechanical trauma (e.g., by forceful passage of a partially inflated balloon catheter within the vessel) or by mechanical trauma in combination with small amounts of a proteolytic enzyme such as dispase, trypsin, collagenase, papain, pepsin, chymotrypsin or cathepsin, or by incubation with these proteolytic enzymes alone. In addition to proteolytic enzymes, lipases may be used. The region of the blood vessel may also be denuded by treatment with a mild detergent or the like, such as NP-40, Triton X100, deoxycholate, or SDS.

The denudation conditions are adjusted to achieve essentially complete loss of endothelium for cell transfers or approximately 20 to 90%, preferably 50 to 75%, loss of cells from the vessel wall for direct infection. In some instances cell removal may not be necessary. The catheter is then advanced so that the instillation port means (3) is placed in the region of denuded endothelium. Infected, transfected or normal cells are then instilled into the discrete section of artery over thirty minutes. If the blood vessel is perfusing an organ which can tolerate some ischemia, e.g., skeletal muscle, distal perfusion is not a major problem, but can be restored by an external shunt if necessary, or by using a catheter which allows distal perfusion. After instillation of the infected endothelial cells, the balloon catheter is removed, and the arterial puncture site and local skin incision are repaired. If distal perfusion is necessary, an alternative catheter designed to allow distal perfusion may be used.

B. Introduction of recombinant genes directly into cells on the wall of a blood vessel or perfused by a specific circulation in vivo; infection or transfection of cells on the vessel wall and organs.

Surgical techniques are used as described above. Instead of using infected cells, a high titer desired genetic material transducing viral vector (10^5 to 10^6 particles/ml) or DNA complexed to a delivery vector is directly instilled into the vessel wall using the double balloon catheter technique. This vector is instilled in medium containing serum and polybrene (10 μ g/ml) to enhance the efficiency of infection. After incubation in the dead space created by the catheter for an adequate period of time (0.2 to 2 hours or greater), this medium is evacuated, gently washed with phosphate-buffered saline, and arterial circulation is restored. Similar protocols are used for post operative recovery.

The vessel surface can be prepared by mechanical denudation alone, in combination with small amounts of proteolytic enzymes such as dispase, trypsin, collagenase or cathepsin, or by incubation with these proteolytic enzymes alone. The denudation conditions are adjusted to achieve the appropriate loss of cells from the vessel wall.

Viral vector or DNA-vector complex is instilled in Dulbecco's modified Eagle's medium using purified virus or complexes containing autologous serum, and adhesive molecules such as polybrene (10 μ g/ml), poly-L-lysine, dextran sulfate, or any polycationic substance which is physiologically suitable, or a hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target in the vessel wall or in the tissue perfused by the vessel to enhance the efficiency of infection by increasing adhesion of viral particles to the relevant target cells. The hybrid antibody directed against the envelope glycoprotein of the virus or the vector and the relevant target cell can be made by one of two methods. Antibodies directed against different epitopes can be chemically crosslinked (G. Jung, C. J. Honsik, R. A. Reisfeld, and H. J. Muller-Eberhard, *Proc. Natl. Acad. Sci. USA*, 83, 4479 (1986); U. D. Staerz, O. Kanagawa, and M. J. Bevan, *Nature*, 314, 628 (1985); and P. Perez, R. W. Hoffman, J. A. Titus, and D. M. Segal, *J. Exp. Med.*, 163, 166 (1986)) or biologically coupled using hybrid hybridomas (U. D. Staerz and M. J. Bevan, *Proc. Natl. Acad. Sci. USA*, 83, 1453 (1986); and C. Milstein and A. C. Cuello, *Nature*, 305, 537 (1983)). After incubation in the central space of the catheter for 0.2 to 2 hours or more, the medium is evacuated, gently washed with phosphate buffered saline, and circulation restored.

Using a different catheter design (see FIG. 2), a different protocol for instillation can also be used. This second approach involves the use of a single balloon means (2) catheter with multiple port means (3) which allow for high pressure delivery of the retrovirus into partially denuded arterial segments. The vessel surface is prepared as described above and defective vector is introduced using similar adhesive molecules. In this instance, the use of a high pressure delivery system serves to optimize the interaction of vectors with cells in adjacent vascular tissue.

The present invention also provides for the use of growth factors delivered locally by catheter or systemically to enhance the efficiency of infection. In addition to retroviral vectors, herpes virus, adenovirus, or other viral vectors are suitable vectors for the present technique.

It is also possible to transform cells within an organ or tissue. Direct transformation of organ or tissue cells may be accomplished by one of two methods. In a first method a high pressure transfection is used. The high pressure will cause the vector to migrate through the blood vessel walls into the surrounding tissue. In a second method, injection into a capillary bed, optionally after injury to allow leaking, gives rise to direct infection of the surrounding tissues.

The time required for the instillation of the vectors or cells will depend on the particular aspect of the invention being employed. Thus, for instilling cells or vectors in a blood vessel a suitable time would be from 0.01 to 12 hrs, preferably 0.1 to 6 hrs, most preferably 0.2 to 2 hrs. Alternatively for high pressure instillation of vectors or cells, shorter times might be preferred.

Obtaining the cells used in this invention

The term "genetic material" generally refers to DNA which codes for a protein. This term also encompasses RNA when used with an RNA virus or other vector based on RNA.

Transformation is the process by which cells have incorporated an exogenous gene by direct infection, transfection or other means of uptake.

The term "vector" is well understood and is synonymous with the often-used phrase "cloning vehicle". A vector is non-chromosomal double-stranded DNA comprising an intact replicon such that the vector is replicated when placed within a unicellular organism, for example by a process of transformation. Viral vectors include retroviruses, adenoviruses, herpesvirus, papovirus, or otherwise modified naturally occurring viruses. Vector also means a formulation of DNA with a chemical or substance which allows uptake by cells.

In another embodiment the present invention provides for inhibiting the expression of a gene. Four approaches may be utilized to accomplish this goal. These include the use of antisense agents, either synthetic oligonucleotides which are complementary to the mRNA (Maher III, L. J. and Dolnick, B. J. *Arch. Biochem. Biophys.*, 253, 214-220 (1987) and Zamecnik, P. C., et al., *Proc. Natl. Acad. Sci.*, 83, 4143-4146 (1986)), or the use of plasmids expressing the reverse complement of this gene (Izant, J. H. and Weintraub, H., *Science*, 229, 345-352, (1985); *Cell*, 36, 1077-1015 (1984)). In addition, catalytic RNAs, called ribozymes, can specifically degrade RNA sequences (Uhlenbeck, O. C., *Nature*, 328, 596-600 (1987), Haseloff, J. and Gerlach, W. L., *Nature*, 334, 585-591 (1988)). The third approach involves "intracellular immunization", where analogues of intracellular proteins can interfere specifically with their function (Friedman, A. D., Triezenberg, S. J. and McKnight, S. L., *Nature*, 335, 452-454 (1988)), described in detail below.

The first approaches may be used to specifically eliminate transcripts in cells. The loss of transcript may be confirmed by S1 nuclease analysis, and expression of binding protein determined using a functional assay. Single-stranded oligonucleotide analogues may be used to interfere with the processing or translation of the transcription factor mRNA. Briefly, synthetic oligonucleotides or thiol-derivative analogues (20-50 nucleotides) complementary to the coding strand of the target gene may be prepared. These antisense agents may be prepared against different regions of the mRNA. They are complementary to the 5' untranslated region, the translational initiation site and subsequent 20-50 base pairs, the central coding region, or the 3' untranslated region of the gene. The antisense agents may be incubated with cells transfected prior to activation. The efficacy of antisense competitors directed at different portions of the messenger RNA may be compared to determine whether specific regions may be more effective in preventing the expression of these genes.

RNA can also function in an autocatalytic fashion to cause autolysis or to specifically degrade complementary RNA sequences (Uhlenbeck, O. C., *Nature*, 328, 596-600 (1987), Haseloff, J. and Gerlach, W. L., *Nature*, 334, 585-591 (1988), and Hutchins, C. J., et al., *Nucleic Acids Res.*, 14, 3627-3640 (1986)). The requirements for a successful RNA cleavage include a hammerhead structure with conserved RNA sequence at the region flanking this structure. Regions adjacent to this cata-

lytic domain are made complementary to a specific RNA, thus targeting the ribozyme to specific cellular mRNAs. To inhibit the production of a specific target gene, the mRNA encoding this gene may be specifically degraded using ribozymes. Briefly, any GUG sequence within the RNA transcript can serve as a target for degradation by the ribozyme. These may be identified by DNA sequence analysis and GUG sites spanning the RNA transcript may be used for specific degradation. Sites in the 5' untranslated region, in the coding region, and in the 3' untranslated region may be targeted to determine whether one region is more efficient in degrading this transcript. Synthetic oligonucleotides encoding 20 base pairs of complementary sequence upstream of the GUG site, the hammerhead structure and ~20 base pairs of complementary sequence downstream of this site may be inserted at the relevant site in the cDNA. In this way, the ribozyme may be targeted to the same cellular compartment as the endogenous message. The ribozymes inserted downstream of specific enhancers, which give high level expression in specific cells may also be generated. These plasmids may be introduced into relevant target cells using electroporation and cotransfection with a neomycin resistant plasmid, pSV2-Neo or another selectable marker. The expression of these transcripts may be confirmed by Northern blot and S1 nuclease analysis. When confirmed, the expression of mRNA may be evaluated by S1 nuclease protection to determine whether expression of these transcripts reduces steady state levels of the target mRNA and the genes which it regulates. The level of protein may also be examined.

Genes may also be inhibited by preparing mutant transcripts lacking domains required for activation. Briefly, after the domain has been identified, a mutant form which is incapable of stimulating function is synthesized. This truncated gene product may be inserted downstream of the SV-40 enhancer in a plasmid containing the neomycin resistance gene (Mulligan, R. and Berg, P., *Science*, 209, 1422-1427 (1980) (in a separate transcription unit). This plasmid may be introduced into cells and selected using G418. The presence of the mutant form of this gene will be confirmed by S1 nuclease analysis and by immunoprecipitation. The function of the endogenous protein in these cells may be evaluated in two ways. First, the expression of the normal gene may be examined. Second, the known function of these proteins may be evaluated. In the event that this mutant intercellular interfering form is toxic to its host cell, it may be introduced on an inducible control element, such as metallothionein promoter. After the isolation of stable lines, cells may be incubated with Zn or Cd to express this gene. Its effect on host cells can then be evaluated.

Another approach to the inactivation of specific genes is to overexpress recombinant proteins which antagonize the expression or function of other activities. For example, if one wished to decrease expression of TPA (e.g., in a clinical setting of disseminate thrombolysis), one could overexpress plasminogen activator inhibitor.

Advances in biochemistry and molecular biology in recent years have led to the construction of "recombinant" vectors in which, for example, retroviruses and plasmids are made to contain exogenous RNA or DNA, respectively. In particular instances the recombinant vector can include heterologous RNA or DNA, by which is meant RNA or DNA that codes for a polypep-

tide ordinarily not produced by the organism susceptible to transformation by the recombinant vector. The production of recombinant RNA and DNA vectors is well understood and need not be described in detail. However, a brief description of this process is included here for reference.

For example, a retrovirus or a plasmid vector can be cleaved to provide linear RNA or DNA having ligatable termini. These termini are bound to exogenous RNA or DNA having complementary like ligatable termini to provide a biologically functional recombinant RNA or DNA molecule having an intact replicon and a desired phenotypical property.

A variety of techniques are available for RNA and DNA recombination in which adjoining ends of separate RNA or DNA fragments are tailored to facilitate ligation.

The exogenous, i.e., donor, RNA or DNA used in the present invention is obtained from suitable cells. The vector is constructed using known techniques to obtain a transformed cell capable of in vivo expression of the therapeutic agent protein. The transformed cell is obtained by contacting a target cell with a RNA- or DNA-containing formulation permitting transfer and uptake of the RNA or DNA into the target cell. Such formulations include, for example, retroviruses, plasmids, liposomal formulations, or plasmids complexes with polycationic substances such as poly-L-lysine, DEAC-dextran and targeting ligands.

The present invention thus provides for the genetic alteration of cells as a method to transmit therapeutic or diagnostic agents to localized regions of the blood vessel for local or systemic purposes. The range of recombinant proteins which may be expressed in these cells is broad and varied. It includes gene transfer using vectors expressing such proteins as tPA for the treatment of thrombosis and restenosis, angiogenesis or growth factors for the purpose of revascularization, and vasoactive factors to alleviate vasoconstriction or vasospasm. This technique can also be extended to genetic treatment of inherited disorders, or acquired diseases, localized or systemic. The present invention may also be used to introduce normal cells to specific sites of cell loss, for example, to replace endothelium damaged during angioplasty or catheterization.

For example, in the treatment of ischemic diseases (thrombotic diseases), genetic material coding for tPA or modifications thereof, urokinase or streptokinase is used to transform the cells. In the treatment of ischemic organ (e.g., heart, kidney, bowel, liver, etc.) failure, genetic material coding for recollateralization agents, such as transforming growth factor α (TGF- α), transforming growth factor β (TGF- β) angiogenin, tumor necrosis factor α , tumor necrosis factor β , acidic fibroblast growth factor or basic fibroblast growth factor can be used. In the treatment of vasomotor diseases, genetic material coding for vasodilators or vasoconstrictors may be used. These include atrial natriuretic factor, platelet-derived growth factor or endothelin. In the treatment of diabetes, genetic material coding for insulin may be used.

The present invention can also be used in the treatment of malignancies by placing the transformed cells in proximity to the malignancy. In this application, genetic material coding for diphtheria toxin, pertussis toxin, or cholera toxin may be used.

In one of its embodiments, the present invention provides for the therapy of malignancy by either stimulat-

ing an immune response against tumor cells or inhibiting tumor cell growth or metastasis by genetic modification in vivo. This approach differs from previous methods in which tumor cells are propagated, modified, and selected in vitro.

In accordance with this embodiment, the present method is used to deliver a DNA sequence or an RNA sequence, including recombinant genes, to tumor cells in vivo with (1) retroviral or viral vectors as vehicles, (2) DNA or RNA/liposome complexes as vehicles, (3) chemical formulations containing the DNA or RNA sequence and coupled to a carrier molecule which facilitates delivery of the sequence to the targeted cells, or (4) by utilizing cell-mediated gene transfer to deliver genes to specific sites in vivo, e.g., by relying upon the use of vascular smooth muscle cells or endothelial cells which have been transduced in vitro as a vehicle to deliver the recombinant gene into the site of the tumor.

In an aspect of this embodiment, the present invention relies on the immune system to provide protection against cancer and play an important role as an adjuvant treatment for a malignancy. Immunotherapy has shown promise as an adjuvant approach to the treatment of malignancies. Both cytolytic T cells and lymphokines can facilitate tumor cell destruction, and strategies to enhance tumor regression by administration of cytokines or tumor infiltrating lymphocytes have shown efficacy in animal models and human trials. For example, it is known that lymphokine activated killer cells (LAK) and tumor infiltrating lymphocytes (TIL) can lyse neoplastic cells and produce partial or complete tumor rejection. Expression of cytokine genes in malignant cells has also enhanced tumor regression.

The present invention provides a novel gene transfer approach against tumors by the introduction of recombinant genes directly into tumor cells in vivo, where, by contrast, traditional gene transfer techniques have focused on modification of tumor cells in vitro followed by transfer of the modified cells. The prior art approaches are disadvantageous because they subject the cells to selection in different growth conditions from those which act in vivo, and because they also require that cell lines be established for each malignancy, thereby rendering adaptability to human disease considerably more difficult.

Genes which may be used with this embodiment include genes containing a DNA sequence (or the corresponding RNA sequence may be used) encoding an intracellular, secreted, or cell surface molecule which is exogenous to the patient and which (1) is immunogenic to the patient, (2) induces rejection, regression, or both, of the tumor, or (3) is toxic to the cells of the tumor.

The vectors containing the DNA sequence (or the corresponding RNA sequence) which may be used in accordance with the invention may be an eukaryotic expression vector containing the DNA or the RNA sequence of interest. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman et al, *Proc. Nat. Acad. Sci. (USA)*, (1987) 84:2150-2154, which is hereby incorporated by reference.

This vector, as noted above, may be administered to the patient in a retroviral or other viral vector (i.e., a viral vector) vehicle, a DNA or RNA/liposome complex, or by utilizing cell-mediated gene transfer. Further, the vector, when present in non-viral form, may be administered as a DNA or RNA sequence-containing chemical formulation coupled to a carrier molecule

which facilitates delivery to the host cell. Such carrier molecule would include an antibody specific to the cells to which the vector is being delivered or a molecule capable of interacting with a receptor associated with the target cells.

Cell-mediated gene transfer may be used in accordance with the invention. In this mode, one relies upon the delivery of recombinant genes into living organisms by transfer of the genetic material into cells derived from the host and modification in cell culture, followed by the introduction of genetically altered cells into the host. An illustrative packaging cell line which may be used in accordance with this embodiment is described in Danos et al, *Proc. Natl. Acad. Sci. (USA)* (1988) 85:6460, which is hereby incorporated by reference.

The DNA or RNA sequence encoding the molecule used in accordance with the invention may be administered to the patient, which may be human or a non-human animal, either locally or systemically. The systemic administration is preferably carried out using the non-viral DNA or RNA chemical formulation coupled to a carrier molecule which facilitates delivery to the host cells. Any of the administrations may be performed by IV or IM injection or subcutaneous injection using any known means, or by the use of the catheter in accordance with the present invention.

The retroviral vector vehicles used in accordance with the present invention comprise a viral particle derived from a naturally-occurring retrovirus which has been genetically altered to render it replication defective and to express a recombinant gene of interest in accordance with the invention. Once the virus delivers its genetic material to a cell, it does not generate additional infectious virus but does introduce exogenous recombinant genes to the cell.

In other viral vectors, the virus particle used is derived from other naturally-occurring viruses which have been genetically altered to render them replication defective and to express recombinant genes. Such viral vectors may be derived from adenovirus, papillomavirus, herpesvirus, parvovirus, etc.

The sequences of the present invention may also be administered as DNA or RNA/liposome complex. Such complexes comprise a mixture of fat particles, lipids, which bind to genetic material, DNA or RNA, providing a hydrophobic coat, allowing genetic material to be delivered into cells. This formulation provides a non-viral vector for gene transfer. Liposomes used in accordance with the invention may comprise DOPE (dioleoyl phosphatidyl ethanol amine), CUDMEDA (N-(5-cholestrum-3- β -ol 3-urethanyl)-N',N'-dimethylethylene diamine).

As noted above, other non-viral vectors may also be used in accordance with the present invention. These include chemical formulations of DNA or RNA coupled to a carrier molecule (e.g., an antibody or a receptor ligand) which facilitates delivery to host cells for the purpose of altering the biologic properties of the host cells. The term "chemical formulations" used herein refers to modifications of nucleic acids to allow coupling of the nucleic acid compounds to a protein or lipid, or derivative thereof, carrier molecule. Such carrier molecules include antibodies specific to the host cells or receptor ligands, i.e., molecules able to interact with receptors associated with the host cells.

The molecules which may be used in accordance with this invention, include the following: (1) genes encoding immune stimulants, such as Class I histocom-

patibility genes, Class II histocompatibility genes, bacterial genes, including mycobacterial (PPD) genes and genes encoding heat shock proteins, viral glycoproteins encoding genes, including vesicular stomatitis virus G protein, influenza hemagglutinin, and herpes virus glycoprotein β , minor histocompatibility antigens, foreign proteins, such as lysozyme or bovine serum albumin, and oncogenes, including E1A, P53 (mutants) and tax; (2) immune and growth stimulants/inhibitors, including inducers of differentiation, such as stimulants, including interleukin-2 (IL-2) IL-4, 3, 6 or 8, inhibitors/inducers of differentiation, such as TNF- α or β , TGF- β (1, 2 or 3), IL-1, soluble growth factor receptors (PDGF, FGF receptors), recombinant antibodies to growth factors or receptors, analogs of growth factors (PDGF, FGF), interferons (α , β or γ) and adhesion molecules; or (3) toxins or negative selectable markers, including thymidine kinase, diphtheria toxin, pertussis toxin or drug-sensitive proteins.

The DNA/RNA sequence is preferably obtained from a source of the same species as the patient, but this is not absolutely required, and the present invention provides for the use of DNA sequences obtained from a source of a species different from the patient in accordance with this embodiment. A preferred embodiment of the present invention, genes encoding immune stimulants and toxins or negative selectable markers, corresponding to (1) and (3) above, are preferably selected from a species different than the species to which the patient belongs. For immune and growth stimulants/inhibitors, corresponding to (2) above, in accordance with another preferred embodiment of the invention, one preferably employs a gene obtained from a species which is the same as the species of the patient.

In the use of the present invention in the treatment of AIDS, genetic material coding for soluble CD4 or derivatives thereof may be used. In the treatment of genetic diseases, for example, growth hormone deficiency, genetic material coding for the needed substance, for example, human growth hormone, is used. All of these genetic materials are readily available to one skilled in this art.

In another embodiment, the present invention provides a kit for treating a disease in a patient which contains a catheter and a solution which contains either an enzyme or a mild detergent, in which the catheter is adapted for insertion into a blood vessel and contains a main catheter body having a balloon element adapted to be inserted into said vessel and expandable against the walls of the blood vessel so as to hold the main catheter body in place in the blood vessel, and means carried by the main catheter body for delivering a solution into the blood vessel, and the solution which contains the enzyme or mild detergent is a physiologically acceptable solution. The solution may contain a proteolytic enzyme, such as dispase, trypsin, collagenase, papain, pepsin, or chymotrypsin. In addition to proteolytic enzymes, liposomes may be used. As a mild detergent, the solution may contain NP-40, Triton X100, deoxycholate, SDS or the like.

Alternatively, the kit may contain a physiological acceptable solution which contains an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material, or bivalent antibodies. This solution may also contain vectors or cells (normal or transformed). In yet another embodiment the kit may contain a catheter and both a solution which contains an enzyme or mild detergent and a solution which contains

an agent such as heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material or bivalent antibody and which may optionally contain vectors or cells.

The kit may contain a catheter with a single balloon and central distal perfusion port, together with acceptable solutions to allow introduction of cells in a specific organ or vectors into a capillary bed or cells in a specific organ or tissue perfused by this capillary bed.

Alternatively, the kit may contain a main catheter body which has two spaced balloon elements adapted to be inserted in a blood vessel with both being expansible against the walls of the blood vessel for providing a chamber in the blood vessel, and to hold the main catheter body in place. In this case, the means for delivering a solution into the chamber is situated in between the balloon elements. The kit may contain a catheter which possesses a plurality of port means for delivering the solution into the blood vessel.

Thus, the present invention represents a method for treating a disease in a patient by causing a cell attached onto the walls of a vessel or the cells of an organ perfused by this vessel in the patient to express an exogenous therapeutic agent protein, wherein the protein treats the disease or may be useful for diagnostic purposes. The present method may be used to treat diseases, such as an ischemic disease, a vasomotor disease, diabetes, a malignancy, AIDS or a genetic disease.

The present method may use exogenous therapeutic agent proteins, such as tPA and modifications thereof, urokinase, streptokinase, acidic fibroblast growth factor, basic fibroblast growth factor, tumor necrosis factor α , tumor necrosis factor β , transforming growth factor α , transforming growth factor β , atrial natriuretic factor, platelet-derived growth factor, endothelin, insulin, diphtheria toxin, pertussis toxin, cholera toxin, soluble CD4 and derivatives thereof, and growth hormone to treat diseases.

The present method may also use exogenous proteins of diagnostic value. For example, a marker protein, such as β -galactosidase, may be used to monitor cell migration.

It is preferred, that the cells caused to express the exogenous therapeutic agent protein be endothelial cells.

Other features of the present invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

The data reported below demonstrate the feasibility of endothelial cell transfer and gene transplantation; that endothelial cells may be stably implanted in situ on the arterial wall by catheterization and express a recombinant marker protein, β -galactosidase, in vivo.

Because atherogenesis in swine has similarities to humans, an inbred pig strain, the Yucatan minipig (Charles River Laboratories, Inc., Wilmington, Mass.), was chosen as an animal model (1). A primary endothelial cell line was established from the internal jugular vein of an 8 month-old female minipig. The endothelial cell identity of this line was confirmed in that the cells exhibited growth characteristics and morphology typical of porcine endothelium in tissue culture. Endothelial cells also express receptors for the acetylated form of low density lipoprotein (AcLDL), in contrast to fibroblasts and other mesenchymal cells (2). When analyzed for ACLDL receptor expression, greater than 99% of

the cultured cells contained this receptor, as judged by fluorescent ACLDL uptake.

Two independent β -galactosidase-expressing endothelial lines were isolated following infection with a murine amphotropic β -galactosidase-transducing retroviral vector (BAG), which is replication-defective and contains both β -galactosidase and neomycin resistance genes (3). Cells containing this vector were selected for their ability to grow in the presence of G-418. Greater than 90% of selected cells synthesized β -galactosidase by histochemical staining. The endothelial nature of these genetically altered cells was also confirmed by analysis of fluorescent ACLDL uptake. Infection by BAG retrovirus was further verified by Southern blot analysis which revealed the presence of intact proviral DNA at approximately one copy per genome.

Endothelial cells derived from this inbred strain, being syngeneic, were applicable for study in more than one minipig, and were tested in nine different experimental subjects. Under general anesthesia, the femoral and iliac arteries were exposed, and a catheter was introduced into the vessel (FIG. 1). Intimal tissues of the arterial wall were denuded mechanically by forceful passage of a partially inflated balloon catheter within the vessel. The artery was rinsed with heparinized saline and incubated with the neutral protease, dispase (50 U/ml), which removed any remaining luminal endothelial cells. Residual enzyme was rapidly inactivated by $\alpha 2$ globulin in plasma upon deflating the catheter balloons and allowing blood to flow through the vessel segment. The cultured endothelial cells which expressed β -galactosidase were introduced using a specially designed arterial catheter (USCI, Billerica, Mass.) that contained two balloons and a central instillation port (FIG. 1).

When these balloons were inflated, a protected space was created within the artery into which cells were instilled through the central port 3 (FIG. 1). These endothelial cells, which expressed β -galactosidase, were allowed to incubate for 30 minutes to facilitate their attachment to the denuded vessel. The catheter was then removed, the arterial branch ligated, and the incision closed.

Segments of the artery inoculated with β -galactosidase-expressing endothelium were removed 2 to 4 weeks later. Gross examination of the arterial specimen after staining using the X-gal chromogen showed multiple areas of blue coloration, compared to an artery seeded with uninfected endothelium, indicative of β -galactosidase activity. Light microscopy documented β -galactosidase staining primarily in endothelial cells of the intima in experimentally seeded vessels.

In contrast, no evidence of similar staining was observed in control segments which had received endothelial cells containing no β -galactosidase. β -Galactosidase staining was occasionally evident in deeper intimal tissues, suggesting entrapment or migration of seeded endothelium within the previously injured vessel wall. Local thrombosis was observed in the first two experimental subjects. This complication was minimized in subsequent studies by administering acetylsalicylic acid prior to the endothelial cell transfer procedure and use of heparin anticoagulation at the time of inoculation. In instances of thrombus formation, β -galactosidase staining was seen in endothelial cells extending from the vessel wall to the surface of the thrombus.

A major concern of gene transplantation *in vivo* relates to the production of replication-competent retrovirus from genetically engineered cells. In these tests, this potential problem has been minimized through the use of a replication defective retrovirus. No helper virus was detectable among these lines after 20 passages *in vitro*. Although defective viruses were used because of their high rate of infectivity and their stable integration into the host cell genome (4), this approach to gene transfer is adaptable to other viral vectors.

A second concern involves the longevity of expression of recombinant genes *in vivo*. Endothelial cell expression of β -galactosidase appeared constant in vessels examined up to six weeks after introduction into the blood vessel in the present study.

These tests have demonstrated that genetically-altered endothelial cells can be introduced into the vascular wall of the Yucatan minipig by arterial catheterization. Thus, the present method can be used for the localized biochemical treatment of vascular disease using genetically-altered endothelium as a vector.

A major complication of current interventions for vascular disease, such as balloon angioplasty or insertion of a graft into a diseased vessel, is disruption of the atherosclerotic plaque and thrombus formation at sites of local tissue trauma (5). In part, this is mediated by endothelial cell injury (6). The present data show that genetically-altered endothelial cells can be introduced at the time of intervention to minimize local thrombosis.

This technique can also be used in other ischemic settings, including unstable angina or myocardial infarction. For instance, antithrombotic effects can be achieved by introducing cells expressing genes for tissue plasminogen activator or urokinase. This technology is also useful for the treatment of chronic tissue ischemia. For example, elaboration of angiogenic or growth factors (7) to stimulate the formation of collateral vessels to severely ischemic tissue, such as the myocardium. Finally, somatic gene replacement for systemic inherited diseases is feasible using modifications of this endothelial cell gene transfer technique.

Another aspect of the present invention relates a method for modulating the immune system of an animal by *in vivo* transformation of cells of the animal with a recombinant gene. The transformation may be carried out either in a non-site-specific or systemic manner or a site-specific manner. If the transformation is carried out in a systemic fashion or at sites other than those which confer specificity on the immune system, such as the thymus, then the immune system will be modulated to result in the animal being sensitized to the molecule for which the recombinant gene encodes. Alternatively, if the transformation is carried out in a site-specific manner and is localized to a site which determines the specificity of the immune system, e.g., the thymus, the immune system will be modulated to result in the animal being tolerized to the molecule encoded by the recombinant gene.

By the term sensitized, it is meant that the immune system exhibits a stronger response to the molecule encoded by the DNA after *in vivo* transformation as compared to before transformation. By the term tolerized, it is meant that the immune system displays a reduced response to the molecule encoded by the recombinant gene after transformation as compared to before transformation. Thus, one may modulate an immune system to provide either a resistance or a tolerance to the molecule encoded by the DNA.

Examples of molecules for which it may be desirable to provide a resistance to include: cell surface molecules, such as tumor antigens (carcinoembryonic antigen), protozoan antigens (pneumocystis), viral antigens (HIV gp120 and gp160, H. influenza antigen, and hepatitis B surface antigen), Lyme disease antigen, Bacterial antigens, and transplantation antigens (Class I or II), ras or other oncogenes, including erb-A or neu; cytoplasmic proteins, such as the raf oncogene, src oncogene, and abl oncogene; nuclear proteins, such as E1A oncogene, mutant p53 oncogene, tat, tax, rev, vpu, vpx, hepatitis core antigen, EBNA and viral genes; and secreted proteins, such as endotoxin, cholera toxin, TNF, and osteoclast activating factor.

Examples of molecules for which it may be desirable to provide a resistance to include: cell surface molecules, such as growth factor receptors, insulin receptors, thyroid hormone receptors, transplantation antigens (class I or II), blood group antigens, and LDL receptor; cytoplasmic proteins, such as cytochrome P450, galactosyl transferase, dystrophin, neomycin resistance gene, and bacterial heat shock protein; nuclear proteins, such as retinoblastoma and transdominant rev; and secreted proteins, such as growth hormone for dwarfs, insulin for diabetics, and adenosine deaminase.

It is to be understood that the nucleic acid, DNA, RNA, or derivative thereof, in the recombinant gene may be of any suitable origin. That is the nucleic acid may be isolated from a naturally occurring source or may be of synthetic origin.

The recombinant gene may be introduced in the cells of the animal using any conventional vector. Such vectors include viral vectors, cationic lipids complexed to DNA or RNA (DNA or RNA/liposomes) and DNA or RNA complexes with polycations, such as DEAE, dextran, and polybrene.

As noted above the recombinant gene can be introduced into cells in a site-specific manner to confer resistance to the molecule encoded by the recombinant gene. Suitable sites include, e.g., endothelial cells or reticuloendothelial cells in the vasculature or any specific tissue or organ. The form of the preparation containing the vector and recombinant gene used in the transformation will depend on the specific tissue to be transformed. Suitable preparations for transforming endothelial cells are described elsewhere in this specification. In addition, preparations suitable for oral or other means of administration (e.g., endoscopic) may be used to provide mucosal resistance. Such preparation could include detergents, gelatins, capsules or other delivery vehicles to protect against degradation and enhance delivery to the mucosal surface, in addition to the vector and gene.

Alternatively, the recombinant gene may be introduced in a site specific fashion to a site which determines the specificity of the immune system. The thymus is such a site (see: A. M. Posselt et al, Science, vol. 249, p. 1292 (1990)). Thus, by introducing a recombinant gene site-specifically into the thymus, the immune system may be modulated to result in a tolerance to the molecule encoded by the gene. In this way, transplant rejection may be suppressed. The same preparations and techniques used to site-specifically transform tumors described above may be used to introduce the recombinant gene into the thymus. Specifically, the transformation preparation may be injected directed into the thymus or tumor or into the vascular supply of the thymus or tumor.

The present method may be practiced on any animal, such as chickens or mammals such as cows, horses, cats, dogs, monkeys, lemurs or humans.

When the recombinant gene is introduced using a liposome, it is preferred to first determine in vitro the optimal values for the DNA: lipid ratios and the absolute concentrations of DNA and lipid as a function of cell death and transformation efficiency for the particular type of cell to be transformed and to use these values in the in vivo transformation. The in vitro determination of these values can be easily carried out using the techniques described in the Experimental Section of this specification.

Another aspect of the present invention relates to a kit for the in vivo systemic introduction of a recombinant gene into cells of an animal. Such a kit would include approximately the optimal amount of a carrier, such as a lipid, and nucleic acid, and/or a means of delivery, e.g., an endoscope or a syringe. The kit may also contain instructions for the administration of the transforming preparation. The carrier and nucleic acid may be freeze dried and may be packaged separately or premixed. The kit may also contain a solution to optimally reconstitute the complexes of the carrier and the nucleic acid, which provide for efficient delivery to cells in vivo. Such a solution may contain one or more ingredients, such as buffers, sugars, salts, proteins, and detergents.

Having generally described the invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Experimental section

A. Analysis of AcLDL receptor expression in normal and β -galactosidase-transduced porcine endothelial cells.

Endothelial cell cultures derived from the Yucatan minipig, two sublines infected with BAG retrovirus or 3T3 fibroblast controls were analyzed for expression of AcLDL receptor using fluorescent labelled AcLDL.

Endothelial cells were derived from external jugular veins using the neutral protease dispase (8). Excised vein segments were filled with dispase (50 U/ml in Hanks' balanced salt solution) and incubated at 30° C. for 20 minutes. Endothelium obtained by this means was maintained in medium 199 (GIBCO, Grand Island, N.Y.) supplemented with fetal calf serum (10%), 50 μ g/ml endothelial cell growth supplement (ECGS) and heparin (100 μ g/ml). These cells were infected with BAG retrovirus, and selected for resistance to G-418. Cell cultures were incubated with (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (DiI) (Biomedical Technologies, Stoughton, Mass.) (10 μ g/ml) for 4-6 hrs. at 37° C., followed by three rinses with phosphate-buffered saline containing 0.5% glutaraldehyde. Cells were visualized by phase contrast and fluorescent microscopy.

B. Method of introduction of endothelial cells by catheterization.

A double balloon catheter was used for instillation of endothelial cells. The catheter has a proximal and distal balloon, each 6 mm in length and 5 mm in width, with a 20 mm length between the balloons. The central section of the catheter has a 2 mm pore connected to an instillation port. Proximal and distal balloon inflation isolates a central space, allowing for instillation of in-

fect cells through the port into a discrete segment of the vessel. For a schematic representation of cell introduction by catheter, see FIGS. 1 and 2.

Animal care was carried out in accordance with "Principles of Laboratory Animal Care" and "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, Revised 1978). Female Yucatan minipigs (80-100 kg) were anesthetized with pentobarbital (20 mg/kg), intubated, and mechanically ventilated. These subjects underwent sterile surgical exposure of the iliac and femoral arteries. The distal femoral artery was punctured, and the double-balloon catheter was advanced by guidewire into the iliac artery. The external iliac artery was identified; the proximal balloon was partially inflated and passed proximally and distally so as to mechanically denude the endothelium. The catheter was then positioned with the central space located in the region of denuded endothelium, and both balloons were inflated. The denuded segment was irrigated with heparinized saline, and residual adherent cells were removed by instillation of dispase (20 U/ml) for 10 min. The denuded vessel was further irrigated with a heparin solution and the BAG-infected endothelial cells were instilled for 30 min. The balloon catheter was subsequently removed, and antegrade blood flow was restored. The vessel segments were excised 2 to 4 weeks later. A portion of the artery was placed in 0.5% glutaraldehyde for five minutes and stored in phosphate-buffered saline, and another portion was mounted in a paraffin block for sectioning. The presence of retroviral expressed β -galactosidase was determined by a standard histochemical technique (19).

C. Analysis of endothelial cells in vitro and in vivo.

β -Galactosidase activity was documented by histochemical staining in (A) primary endothelial cells from the Yucatan minipig, (B) a subline derived by infection with the BAG retroviral vector, (C) a segment of normal control artery, (D) a segment of artery instilled with endothelium infected with the BAG retroviral vector, (E) microscopic cross-section of normal control artery, and (F) microscopic cross-section of artery instilled with endothelium infected with the BAG retroviral vector.

Endothelial cells in tissue culture were fixed in 0.5% glutaraldehyde prior to histochemical staining. The enzymatic activity of the *E. coli* β -galactosidase protein was used to identify infected endothelial cells in vitro and in vivo. The β -galactosidase transducing Mo-MuLV vector (2), (BAG) was kindly provided by Dr. Constance Cepko. This vector used the wild type Mo-MuLV LTR as a promoter for the β -galactosidase gene. The simian virus 40 (SV-40) early promoter linked to the Tn5 neomycin resistance gene provides resistance to the drug G-418 and is inserted downstream of the β -galactosidase gene, providing a marker to select for retrovirus-containing, β -galactosidase expressing cells. This defective retrovirus was prepared from fibroblast ψ am cells (3,10), and maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% calf serum. Cells were passaged twice weekly following trypsinization. The supernatant, with titers of 10^4 - 10^5 /ml G-418 resistant colonies, was added to endothelial cells at two-thirds confluence and incubated for 12 hours in DMEM with 10% calf serum at 37° C. in 5% CO₂ in the presence of 8 μ g/ml of polybrene. Viral supernatants were removed, and cells maintained in medium 199 with 10% fetal calf serum, ECGS (50 μ g/ml), and endothelial cell conditioned medium (20%)

for an additional 24 to 48 hours prior to selection in G-418 (0.7 µg/ml of a 50% racemic mixture). G-418 resistant cells were isolated and analyzed for β -galactosidase expression using a standard histochemical stain (9). Cells stably expressing the β -galactosidase enzyme were maintained in continuous culture for use as needed. Frozen aliquots were stored in liquid nitrogen.

D. Immunotherapy of Malignancy by In Vivo Gene Transfer.

A retroviral vector which the H-2K^S gene was prepared. CT26 cells were infected with this vector in vitro, selected for G418 resistance, and analyzed by fluorescence activated cell sorting (FACS). Transduced CT26 cells showed a higher mean fluorescence intensity than uninfected CT26 cells or CT26 infected with different retroviral vectors. When 10⁶ CT26 cells which express H-2K^S were injected subcutaneously into BALB/c mice (H-2^d) sensitized to this antigen, no tumors were observed over an 8-week period in contrast to the unmodified CT26 (H-2^d) tumor line which routinely formed tumors at this dose. The immune response to H-2K^S could therefore provide protection against CT26 cells bearing this antigen. When CT26 H-2K^S and CT26 were co-inoculated, however, tumor growth was observed, suggesting that H-2K^S conferred sensitivity only to modified cells.

To determine whether protective effects could be achieved by introduction of H-2K^S in growing CT26 tumors, the recombinant H-2K^S reporter or a β -galactosidase gene was introduced into tumors either with a DNA/liposome or a retroviral vector. Tumor capsules (0.5-1 cm diameter) were exposed surgically and multiple needle injections (2-10) delivered to the parenchyma. With β -galactosidase reporter plasmids, recombinant gene expression could be readily detected after intra-tumor injection of DNA/liposome or retroviral vectors.

In mice which received intra-tumor injections of the H-2K^S DNA/liposome complex or H-2K^S retroviral vector, the recombinant DNA was detected by PCR in the tumor and occasionally in other tissues. When found in the other organs, no evidence of inflammation or organ toxicity was detected pathologically. An immune response to the recombinant H-2K^S protein was evident in these animals, however. Lymphocytes derived from the H-2K^S, but not β -galactosidase transduced tumors, demonstrated a cytolytic response to H-2K^S whether delivered by retroviral vectors or liposomes. More importantly, lymphocytes derived from the H-2K^S, but not β -galactosidase transduced animals, recognized and lysed unmodified CT26 cells, indicating that this stimulation induced immune reactivity against genetically unmodified tumor cells.

To assess the protective effect of the immune response against H-2K^S, tumor growth in vivo was quantitated. When animals received no prior sensitization to H-2K^S, one of four tumors transduced with H-2K^S showed attenuation of tumor growth which was not complete. In contrast, no anti-tumor effect was seen in unmodified (n=4) or β -galactosidase transduced controls (n=4). Because these tumors were large at the time of initial injection and continued to grow as the primary immune response was generated, an attempt was made to optimize the anti-tumor response by pre-immunization of mice with irradiated CT26 H-2K^S tumor cells, and by earlier and/or more frequent injections of vector. Tumors were transduced on days 12 and 32 by intra-tumor injection of H-2K^S or β -galactosi-

dase DNA/liposome vectors. Treatment with the H-2K^S liposome complex improved survival and attenuated tumor growth, in contrast to β -galactosidase transduced tumors where there was no difference in growth rate compared to the uninjected controls. Complete tumor regression was achieved in two mice by increasing the number of injections and by delivery of H-2K^S into tumors at an earlier stage. This treatment was protective, since control animals showed continued tumor growth and did not survive beyond 35 days.

E. Modulation of the Immune System.

The response to injection of cationic lipids and plasmids was determined after injection intravenously into BALB/c mice (6-12 weeks). In the first experiments, a gene encoding the H-2K^S molecule was introduced by tail vein injection. Two to four weeks later, spleen cells were harvested and analyzed for their ability to mediate a cytolytic T cell response. When these cells were tested using ⁵¹Cr target cells (CT26 cells expressing the H-2K^S gene), significant cytotoxicity was observed which was not seen in animals injected with the control vector, β -galactosidase (see FIG. 3). Up to 25% of target cells were lysed at effector: target ratios of 25:1.

In addition to this specific cytolytic T cell response, serologic or antibody responses to genes encoded by expression vector plasmids have been examined. When a plasmid encoding the gp160 molecule of HIV is injected, an antibody response is elicited in treated mice. In contrast to control animals injected with cationic lipids containing β -galactosidase, mice injected with cationic lipids with gp 160 plasmid showed an antibody response to the gp160 and gp120 form of this molecule by Western blot analysis (See FIG. 4). These results demonstrate that systemic administration of cationic lipid/DNA complexes can be used successfully to induce cell-mediated and antibody-mediated immunity against foreign pathogens.

F. Determination of Optimal Transfection Conditions.

(1) Plasmid Construction

A plasmid containing the *E. coli* lacZ gene under the control of the Rous Sarcoma Virus LTS (RSV- β -gal) (Norton and Coffin, *Mol. Cell Biol.*, 5(2), 281-290, 1985) was used for transfection of porcine primary endothelial and HeLa cells. In addition, a plasmid containing the lacZ gene under the control of preproendothelin-1 5'-flanking DNA (-1410 to +83) (Wilson et al., *Mol. Cell Biol.*, 10(9), 4854-4862, 1990) was used for transfection of endothelial cells. For in vivo toxicity analysis, the RSV- β -gal plasmid, and a plasmid derived from the PLJ vector containing the cDNA encoding an H-2K^S mouse MHC class I gene were used.

(2) Cell Culture, Transfection Analysis, and Toxicity in Vitro

Primary endothelial cells, derived from the Yucatan minipig (YPE cells), were incubated with medium 199 (M199) supplemented with 10% FBS, 2 mM l-glutamine, 50 U/ml penicillin, and 5 µg/ml streptomycin. HeLa cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 5% FBS, 2 mM l-glutamine, 50 U/ml penicillin and 5 µg/ml streptomycin. The DNA liposome mixture was prepared with lipid concentrations of DOPE/DC-Chol between 2.5 and 25 µM added to 0.2 ml of serum-free media or Ringer's lactate solution in polystyrene tubes. After mixing gently, the solution was allowed to stand at room temperature for 15-20 minutes. For transfection analysis, cells were grown in 60 mm tissue culture

dishes at 75% confluency or greater. Cells were washed twice with serum-free media or lactated Ringers solution and then placed in 0.5 mls of the same media. The DNA liposome solution (0.2 ml) was then added slowly to the cells, with gentle mixing, with a final volume of 0.7 ml. This resulted in DNA concentrations between 0.7 and 7 $\mu\text{g}/\text{ml}$ (13–130 nM), and lipid concentrations of 7–70 μM . Transfection was allowed to proceed for 1–5 hours, after which the cells were placed in media supplemented as described above. At 24–48 hours after transfection the enzymatic activity of the *E. coli* β -galactosidase protein was used to identify transfected cells by staining with the X-gal chromagen. Toxicity in vitro was assessed by cytopathic effect or trypan blue exclusion.

(3) Animal Studies

For intravenous injections, the DNA/liposomes were prepared as described for the in vitro transfection studies in 0.2 ml of serum-free M199 or lactated-Ringers solution. After 15–20 min of incubation, the mixture was diluted to 0.7 ml and 0.1 to 0.2 ml of this dilution was then injected immediately into the tail vein of adult, female BALB/c mice. Blood was collected before injection and 9–11 days following injection, and serum chemistries were examined. At ~2–3 weeks following injection, the liver, kidney, lung, heart, and brain were extracted for histologic and PCR DNA amplification analysis as described previously. Intratumor injection of CT26 cells (Fearon et al., *Cell*, 60, 397–403, 1990) and analysis were also performed according to the previous protocols.

(4) Results

The optimal conditions for transfection and toxicity of DNA/liposomes were initially determined in vitro. To obtain maximal transfection without toxicity in vitro, the ratio of DNA to cationic lipid, the absolute concentration of DNA or lipids, and the conditions for mixture of DNA and cationic lipids were studied. The cationic lipid preparation was a formulation of two compounds, which include dioleoyl phosphatidylethanolamine (DOPE) and cholesten-3- β -ol 3-urethanyl-N',N' dimethylethylene diamine (DC-chol). The transfection efficiencies of this reagent were equal to or greater than those of Lipofectin® (BRL) in several cell lines in vitro. Endothelial cells, which are typically difficult to transfect, and HeLa cells, which can be transfected easily using a variety of techniques, were examined by transfection in vitro.

To determine the optimal conditions for transfection of endothelial cells, the lipid was initially used at different concentrations while the DNA concentration was held constant. Maximal transfection efficiency was seen using 0.7 $\mu\text{g}/\text{ml}$ DNA (13 nM) and 21 μM of DOPE/DCChol lipid, with a sharp decline in the number of transfected cells with higher or lower lipid concentrations. Next, the DNA concentration was altered as the lipid concentration remained constant. This analysis revealed a similar sensitivity to DNA concentration, with the number of transfected cells decreasing significantly with increments of DNA concentration as low as 0.4 $\mu\text{g}/\text{ml}$. These results indicate that the ratio of DNA to lipid is important for maximum transfection efficiency, and that the absolute concentration of each component is also important in determining the efficiency of transfection. An increase in DNA and lipid concentration beyond the optimal concentration of 0.7 $\mu\text{g}/\text{ml}$ DNA (13 nM) and 21 μM of DOPE/DC-Chol reduced the number of viable cells and did not increase

the transfection efficiency of the remaining viable cells. Lipid concentrations greater than 35 μM reduced the number of viable cells by 50% compared to the untransfected control, whereas the optimal concentration of 0.7 $\mu\text{g}/\text{ml}$ DNA (13 nM) and 21 μM of lipid had no effect on cell viability after 5 hours of incubation.

To compare the optimal concentrations of transfection in a different cell type, transfections were performed on HeLa cells. In this case, a slightly different optimal ratio of DNA and lipid were observed. Peak transfection efficiencies were obtained at the same lipid concentration as endothelial cells (21 $\mu\text{g}/\text{ml}$) but varied less with small differences in DNA concentrations. DNA concentrations of 1.4–4.2 $\mu\text{g}/\text{ml}$ were equally effective. Again, when the ratio of DNA to lipid was maintained but the concentration of each was decreased three-fold, very few cells were transfected, illustrating that both the ratio of DNA to lipid and the absolute concentration of each component are important in maximizing the number of transfected cells. If HeLa cells were transfected at >80% confluence or greater, there was no toxicity using up to 35 μM of lipid. When cells were transfected at a lower saturation density, however, cell viability was reduced dramatically with as little as 7 μM of lipid compared to the untransfected control cells. These results demonstrate that the optimal conditions for transfection and toxicity may differ somewhat depending on the cell line.

Another variable in the preparation of liposomes was the composition of the solution used to generate complexes of the cationic lipids with DNA. Among several media solutions analyzed, no substantial difference was noted in transfection efficiency or toxicity with M199, McCoy's, OptiMEM, or RPMI media. A significant improvement in transfection efficiency was observed, however, using standard Ringers lactate. The number of transfected cells increased more than 3-fold compared to the serum-free medium, although prolonged incubation (≥ 2 hours) resulted in a loss of cell viability in some cell types.

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Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

We claim:

1. A kit for treating a disease in a patient in need thereof, comprising a catheter and a physiologically acceptable solution, wherein:
 - (i) said catheter is adapted for insertion into a blood vessel and comprises a main catheter body having a balloon element, adapted to be inserted in said blood vessel and being expansible against the walls of said vessel so as to hold said main catheter body in place, and means carried by said main catheter body for delivering said solution into said blood vessel;
 - (ii) said physiologically acceptable solution comprises DNA and at least one member selected from the group consisting of heparin, poly-L-lysine, polybrene, dextran sulfate, a polycationic material, and bivalent antibodies.
2. The kit of claim 1, wherein said physiologically acceptable solution further comprises a growth factor.

3. A kit for treating a disease in a patient in need thereof, comprising
 - (i) a catheter adapted for insertion into a blood vessel, comprising a main catheter body having a balloon element adapted to being inserted into said vessel and expansible against the walls of the said vessels so as to hold said main catheter body in place in said vessel and a means carried by said main catheter body for delivering a physiologically acceptable solution into said blood vessel;
 - (ii) said physiologically acceptable solution which may contain an enzyme, mild detergent or lipid; and
 - (iii) a means for causing a cell attached onto the walls of a vessel or in an organ or tissue in said patient to express an exogenous therapeutic agent protein, comprising a formulation adapted for delivery by said catheter for the transfer and uptake of RNA or DNA into said cell attached onto the walls of a vessel or in an organ or tissue in said patient.
4. The kit according to claim 3, wherein said DNA is antisense DNA.
5. The kit of claim 3, wherein said solution contains, as said enzyme, at least one member selected from the group consisting of dispase, trypsin, collagenase, papain, pepsin, chymotrypsin, and lipases.
6. The kit of claim 3, wherein said solution contains at least one member selected from the group consisting of Nonidet P-40, Triton X100, deoxycholate, and sodium dodecyl sulfate.
7. The kit of claim 3, wherein said main catheter body comprises two spaced balloon elements, adapted to be inserted in a blood vessel and both being expansible against the walls of the blood vessel, for providing a chamber in said blood vessel and so as to hold said main catheter body in place, and whereas said means for delivering a physiologically acceptable solution into said chamber is situated in between said balloon elements.
8. The kit of claim 3, wherein said means for delivering said solution into said blood vessel comprises a plurality of pore means.
9. The kit of claim 3, wherein said formulation comprises a retrovirus, a plasmid, a liposomal formulation, or a plasmid complex with a polycationic substance.
10. The kit of claim 3, wherein said formulation is a liposomal formulation.

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UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,328,470

Page 1 of 2

DATED : July 12, 1994

INVENTOR(S) : Elizabeth G. Nabel et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1: line 25, "would an asset"
should read --would be an asset--.

Column 7: line 17, "cells though"
should read --cells through--.

Column 21: line 39, "DNA/liposomes comples"
should read --DNA/liposomes complex--;

line 41, "occasioinally"
should read --occasionally--;

line 43, "ogran"
should read --organ--;

line 68, "injuection"
should read --injection--.

Column 22: line 27, "plasmnid"
should read --plasmid--.

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It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 23: line 65, "incrase"
should read --increase--.

Column 24: line 5, "ande"
should read --and--.

Column 25: line 25, "that. within"
should read --that within--.

Signed and Sealed this
Twenty-sixth Day of March, 1996

Attest:



BRUCE LEHMAN

Commissioner of Patents and Trademarks

Attesting Officer

EVIDENCE APPENDIX

ITEM NO. 5

Orlic et al. publication entitled, “Mobilized bone marrow cells repair the infarcted heart, improving function and survival,” (August 28, 2001, PNAS USA, 98:10344-10349) and

Orlic et al. publication entitled, “Bone marrow cells regenerate infarcted myocardium,” (April 5, 2001, Nature, 410:701-705) cited on page 20 in Examiner’s June 1, 2004, Final Office Action

Mobilized bone marrow cells repair the infarcted heart, improving function and survival

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Edited by Eugene Braunwald, Partners HealthCare System, Inc., Boston, MA, and approved June 29, 2001 (received for review April 11, 2001)

Attempts to repair myocardial infarcts by transplanting cardiomyocytes or skeletal myoblasts have failed to reconstitute healthy myocardium and coronary vessels integrated structurally and functionally with the remaining viable portion of the ventricular wall. The recently discovered growth and transdifferentiation potential of primitive bone marrow cells (BMC) prompted us, in an earlier study, to inject in the border zone of acute infarcts Lin⁻ c-kit^{POS} BMC from syngeneic animals. These BMC differentiated into myocytes and vascular structures, ameliorating the function of the infarcted heart. Two critical determinants seem to be required for the transdifferentiation of primitive BMC: tissue damage and a high level of pluripotent cells. On this basis, we hypothesized here that BMC, mobilized by stem cell factor and granulocyte-colony stimulating factor, would home to the infarcted region, replicate, differentiate, and ultimately promote myocardial repair. We report that, in the presence of an acute myocardial infarct, cytokine-mediated translocation of BMC resulted in a significant degree of tissue regeneration 27 days later. Cytokine-induced cardiac repair decreased mortality by 68%, infarct size by 40%, cavity dilation by 26%, and diastolic stress by 70%. Ejection fraction progressively increased and hemodynamics significantly improved as a consequence of the formation of 15×10^6 new myocytes with arterioles and capillaries connected with the circulation of the unaffected ventricle. In conclusion, mobilization of primitive BMC by cytokines might offer a noninvasive therapeutic strategy for the regeneration of the myocardium lost as a result of ischemic heart disease and, perhaps, other forms of cardiac pathology.

Sudden occlusion of a major coronary artery and acute myocardial ischemia lead to rapid death of myocytes (M) and vascular structures in the supplied region of the ventricle. Despite the demonstration that a subpopulation of cardiac muscle cells is able to replicate (1), and new vessels are formed (2), this regeneration is restricted to the viable myocardium. The loss of M, arterioles, and capillaries in the infarcted area is irreversible, resulting with time in the formation of scarred tissue. For this reason, most experimental and clinical therapies have mainly focused on limiting infarct size. Attempts to replace the necrotic zone of the heart by transplanting cardiomyocytes or skeletal myoblasts (3–7), although successful in the survival of many of the grafted cells, have invariably failed to reconstitute healthy myocardium and coronary vessels integrated structurally and functionally with the spared ventricular wall.

The recognition that stem cells, particularly those from the bone marrow, have the capacity to colonize different tissues, proliferate, and transdifferentiate into cell lineages of the host organ (8, 9), prompted us in an earlier study (10) to inject Lin⁻ c-kit^{POS} bone marrow cells (BMC) in the contracting myocardium bordering acute infarcts. Surprisingly, the implanted BMC differentiated into M and coronary vessels ameliorating the function of the injured heart (10). This approach, however, required a surgical intervention that was accompanied by high mortality and a grafting success rate of 40%. Therefore, the identification and utilization of a noninvasive method would be highly desirable. Two main determinants seem to be critical for colonization and transdifferentiation of BMC into a variety of

tissues: recent damage and a high number of circulating stem cells (8, 9, 11, 12). On this basis, we hypothesized that a sufficient number of BMC mobilized by stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF; refs. 13 and 14) would home to the infarcted heart and promote cardiac repair. To test this possibility, mice were injected with SCF and G-CSF to increase the number of circulating stem cells from 29 in nontreated controls to 7,200 in cytokine-treated mice (13).

Materials and Methods

Myocardial Infarction (MI) and Cytokines. C57BL/6 male mice at 2 months of age were splenectomized and 2 weeks later were injected s.c. with recombinant rat SCF, 200 μ g/kg/day, and recombinant human G-CSF, 50 μ g/kg/day (Amgen Biologicals), once a day for 5 days (13, 14). Under ether anesthesia, the left ventricle (LV) was exposed and the coronary artery was ligated (10, 15, 16). SCF and G-CSF were given for 3 more days. Controls consisted of splenectomized infarcted and sham-operated (SO) mice injected with saline. BrdUrd, 50 mg/kg body weight, was given once a day for 13 days before the mice were killed; mice were killed at 27 days. Protocols were approved by New York Medical College.

Echocardiography and Hemodynamics. Echocardiography was performed in conscious mice by using a Sequoia 256c (Acuson, Mountain View, CA) equipped with a 13-MHz linear transducer (15L8). The anterior chest area was shaved and two-dimensional (2D) images and M-mode tracings were recorded from the parasternal short axis view at the level of papillary muscles. From M-mode tracings, anatomical parameters in diastole and systole were obtained (17). Ejection fraction (EF) was derived from LV cross-sectional area in 2D short axis view (17): $EF = [(LVDA - LVSA)/LVDA] \times 100$, where LVDA and LVSA correspond to LV areas in diastole and in systole. Mice were anesthetized with chloral hydrate (400 mg/kg body weight, i.p.), and a microtip pressure transducer (SPR-671; Millar Instruments, Houston) connected to a chart recorder was advanced into the LV for the evaluation of pressures and + and - dP/dt in the closed-chest preparation (10, 15, 16).

Cardiac Anatomy and Infarct Size. After hemodynamic measurements, the abdominal aorta was cannulated, the heart was arrested in diastole with CdCl₂, and the myocardium was perfused with 10% (vol/vol) formalin. The LV chamber was filled with fixative at a pressure equal to the *in vivo* measured

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: BMC, bone marrow cells; SCF, stem cell factor; G-CSF, granulocyte-colony stimulating factor; LV, left ventricle; EC, endothelial cells; SMC, smooth muscle cells; EF, ejection fraction; SO, sham-operated; LVFW, LV free wall; M, myocyte; MI, myocardial infarction.

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end-diastolic pressure (15, 16). The LV intracavitary axis was measured, and three transverse slices from the base, mid-region, and apex were embedded in paraffin. The mid-section was used to measure LV thickness, chamber diameter, and volume (15, 16). Infarct size was determined by the number of M lost from the left ventricular free wall (LVFW; refs. 18 and 19).

Newly Formed M. The volume of regenerating myocardium was determined by measuring in each of three sections the area occupied by the restored tissue and section thickness. The product of these two variables yielded the volume of tissue repair in each section. Values in the three sections were added, and the total volume of formed myocardium was obtained. Additionally, the volume of 400 M was measured in each heart. Sections were stained with desmin and laminin Abs and propidium iodide (PI). Only longitudinally oriented cells with centrally located nuclei were included. The length and diameter across the nucleus were collected in each M to compute cell volume, assuming a cylindrical shape (18, 19). M were divided in classes, and the number of M in each class was calculated from the quotient of total M class volume and average cell volume (20, 21). The number of arteriole and capillary profiles per unit area of myocardium was measured as described (18, 19).

BrdUrd and Ki67. Sections were incubated with BrdUrd or Ki67 Ab. M were recognized with a mouse monoclonal anti-cardiac myosin, endothelial cells (EC) were recognized with rabbit polyclonal anti-factor VIII, and smooth muscle cells (SMC) were recognized with a mouse monoclonal anti- α -smooth muscle actin. The fractions of M, EC, and SMC nuclei labeled by BrdUrd and Ki67 were obtained by confocal microscopy (10). Nuclei sampled in 11 cytokine-treated mice for BrdUrd were M = 3,541; EC = 2,604; SMC = 1,824; and for Ki67 were M = 3,096; EC = 2,465; SMC = 1,404.

Cell Differentiation. Cytoplasmic and nuclear markers were used; M nuclei, rabbit polyclonal Csx/Nkx2.5, MEF2, and GATA4 Abs (10, 22, 23); cytoplasm, mouse monoclonal nestin (24), rabbit polyclonal desmin (25), cardiac myosin, mouse monoclonal α -sarcomeric actin, and rabbit polyclonal connexin 43 Abs (10); EC cytoplasm, mouse monoclonal flk-1, vascular endothelial (VE)-cadherin, and factor VIII Abs (10, 26, 27); and SMC cytoplasm, flk-1 and α -smooth muscle actin Abs (10, 28). Scar was detected by a mixture of collagen type I and type III Abs.

Statistics. Results are mean \pm SD. Significance was determined by the Student's *t* test and Bonferroni method (16). Mortality was computed with a log-rank test. $P < 0.05$ was significant.

Results

BMC Mobilization by Cytokines Reduces Mortality and Induces Myocardial Repair After Infarction. Given the ability of bone marrow $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells to transdifferentiate into the cardiogenic lineage (10), we used a protocol to maximize their number in the peripheral circulation to increase the probability of their homing to the region of dead myocardium. In normal animals, the frequency of $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells in the blood is only a small fraction of similar cells present in the bone marrow (13, 14). We have documented previously that the cytokine treatment used here promotes a marked increase of $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells in the bone marrow and a redistribution of these cells from the bone marrow to the peripheral blood. This protocol leads to a 250-fold increase in $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells in the circulation (13, 14).

In the current study, BMC mobilization by SCF and G-CSF resulted in a dramatic increase in survival of infarcted mice; with cytokine treatment, 73% of mice (11 of 15) survived 27 days, whereas mortality was very high in untreated infarcted mice (Fig. 1A). A large number of animals in this group died from 3 to 6 days

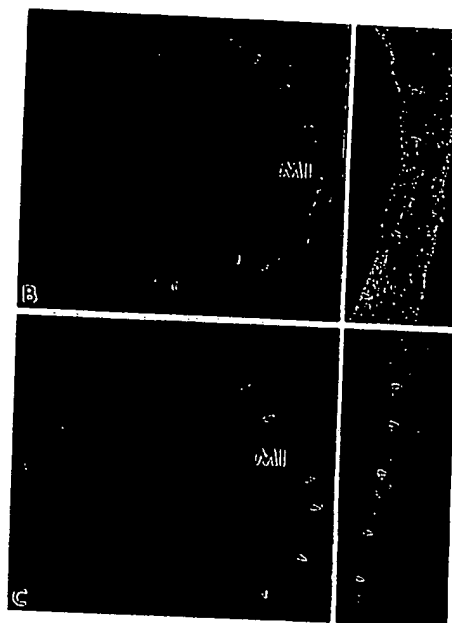
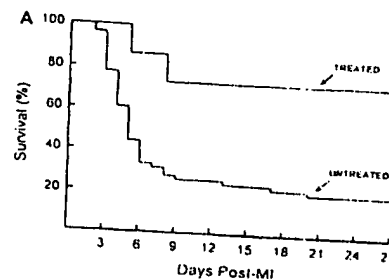


Fig. 1. Mortality and myocardial regeneration. (A) Cytokine-treated infarcted mice, $n = 15$; untreated infarcted mice, $n = 52$; log-rank test, $P < 0.0001$. (B) Large infarct (MI) in a cytokine-treated mouse; forming myocardium (arrowheads) at higher magnification (adjacent panel). (C) MI in a nontreated mouse. Healing comprises the entire infarct (arrowheads). Scar at higher magnification (adjacent panel). Red = cardiac myosin; yellow-green = propidium iodide (PI) labeling of nuclei; blue-magenta = collagen types I and III. (B and C, $\times 20$; Insets, $\times 80$.)

after MI and only 17% (9 of 52) reached 27 days ($P < 0.001$). Mice that died within 48 h post-MI were not included in the mortality curve to minimize the influence of the surgical trauma. Infarct size was similar in the cytokine- [64 \pm 11% ($n = 11$)] and saline- [62 \pm 9% ($n = 9$)] injected animals as measured by the number of M lost in the LVFW at 27 days (see Fig. 5, which is published as supplemental data on the PNAS web site, www.pnas.org).

BMC mobilization promoted myocardial regeneration in all 11 cytokine-treated infarcted mice, killed 27 days after surgery (Fig. 1B). Myocardial growth within the infarct was also seen in the 4 mice that died prematurely at day 6 ($n = 2$) and at day 9 ($n = 2$). Cardiac repair was characterized by a band of newly formed myocardium occupying most of the damaged area. The developing tissue extended from the border zone to the inside of the injured region and from the endocardium to the epicardium of the LVFW. In the absence of cytokines, myocardial replacement was never observed, and healing with scar formation was apparent (Fig. 1C). Conversely, only small areas of collagen accumulation were detected in treated mice.

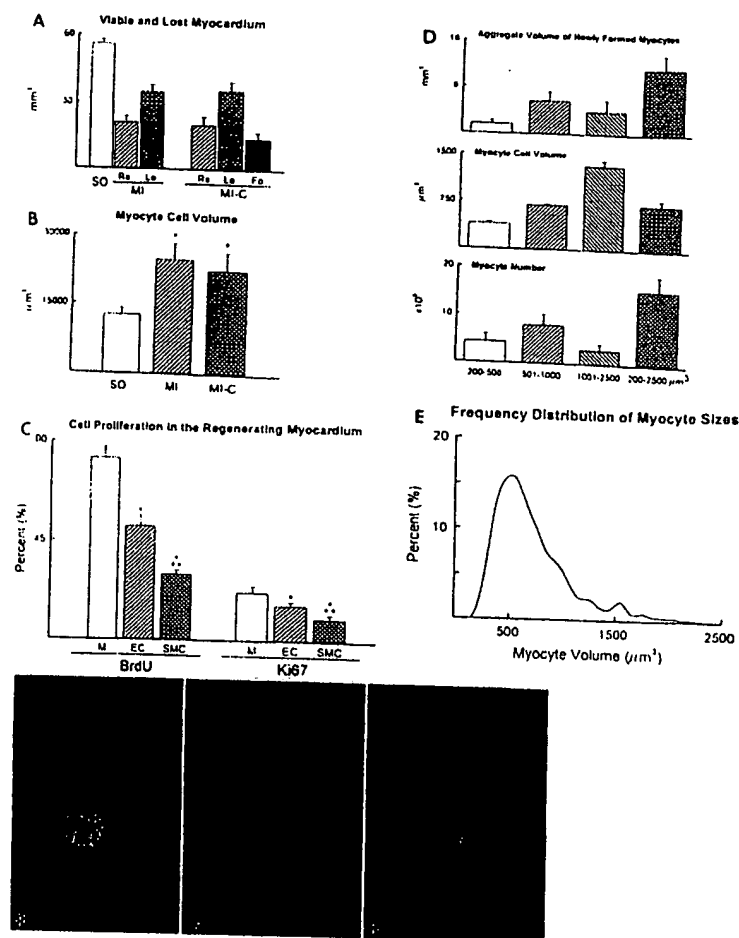


Fig. 2. Myocardial regeneration. (A) Remaining viable (Re), lost (Lo), and newly formed (Fo) myocardium in LVFW at 27 days in MI and MI-C; SO, myocardium without infarct. (B) Cellular hypertrophy in spared myocardium. (C) M, EC, and SMC labeled by BrdUrd and Ki67; $n = 11$. * and **, $P < 0.05$ vs. M and EC. (D and E) Volume, number ($n = 11$), and class distribution (bucket size, $100 \mu\text{m}^3$; $n = 4,400$) of M within the formed myocardium. (F–H) Arterioles with TER-119-labeled erythrocyte membrane (green fluorescence); blue fluorescence = propidium iodide (PI) staining of nuclei; red fluorescence = α -smooth muscle actin in SMC. F, $\times 800$; G and H, $\times 1,200$.

BMC Mobilization Partially Restored Myocardial Mass. To quantify the contribution of the developing band to the ventricular mass, we first determined the volume of the LVFW (weight divided by 1.06 g/ml) in each group of mice. These data were $56 \pm 2 \text{ mm}^3$ in SO, $62 \pm 4 \text{ mm}^3$ (viable FW = 41 ± 3 ; infarcted FW = 21 ± 4) in infarcted nontreated animals, and $56 \pm 9 \text{ mm}^3$ (viable FW = 37 ± 8 ; infarcted FW = 19 ± 5) in infarcted cytokine-treated mice. These values were compared with the expected values of spared and lost myocardium at 27 days, given the size of the infarct in the nontreated and cytokine-treated animals. From the volume of the LVFW (56 mm^3) in SO and infarct size in nontreated (62%) and treated (64%) mice, it was possible to calculate the volume of myocardium destined to remain (nontreated = 21 mm^3 ; treated = 20 mm^3) and destined to be lost (nontreated = 35 mm^3 ; treated = 36 mm^3) 27 days after coronary occlusion (Fig. 2A). The volume of newly formed myocardium was detected exclusively in cytokine-treated mice and found to be 14 mm^3 (Fig. 2A). Thus, the repair band reduced infarct size from 64% ($36 \text{ mm}^3/56 \text{ mm}^3 = 64\%$) to 39% [$(36 \text{ mm}^3 - 14 \text{ mm}^3)/56 \text{ mm}^3 = 39\%$]. Because the spared portion of the LVFW at 27 days was 41 and 37 mm^3 in nontreated and treated mice (see above), the remaining myocardium, shown in Fig. 2A, underwent 95% ($P < 0.001$) and 85% ($P < 0.001$) hypertrophy, respectively. Consistently, M cell volume increased 94% and 77% (Fig. 2B).

Myocardial Regeneration Is Characterized by Dividing Myocytes and Forming Vascular Structures. Ki67 and BrdUrd were used to evaluate the growth stage of the cells in the regenerating band

(Fig. 6 A–D, which is published as supplemental data on the PNAS web site). BrdUrd was injected daily between days 14–26 to measure the cumulative extent of cell proliferation while Ki67 was assayed to determine the number of cycling cells at the time of death. Ki67 identifies cells in G_1 , S , G_2 , prophase, and metaphase, decreasing in anaphase and telophase (10). The percentages of BrdUrd- and Ki67-positive M were 1.6- and 1.4-fold higher than EC, and 2.8- and 2.2-fold higher than SMC, respectively (Fig. 2C). The forming myocardium occupied $76 \pm 11\%$ of the infarct; M constituted $61 \pm 12\%$, new vessels $12 \pm 5\%$, and other components $3 \pm 2\%$. The band contained 15×10^6 regenerating M that were in an active growing phase and had a wide size distribution (Fig. 2D and E). EC and SMC growth resulted in the formation of 15 ± 5 arterioles and 348 ± 82 capillaries per mm^2 of new myocardium. Thick wall arterioles with several layers of SMC and luminal diameters of $10\text{--}30 \mu\text{m}$ represented vessels in early differentiation. At times, incomplete perfusion of the coronary branches within the repairing myocardium during the fixation procedure led to arterioles and capillaries containing erythrocytes (Fig. 2F–H). This observation provided evidence that the new vessels were functionally competent and connected with the coronary circulation. Therefore, tissue repair reduced infarct size and M growth exceeded angiogenesis; muscle mass replacement was the prevailing feature of the infarcted heart.

Five cytoplasmic proteins were identified to establish the state

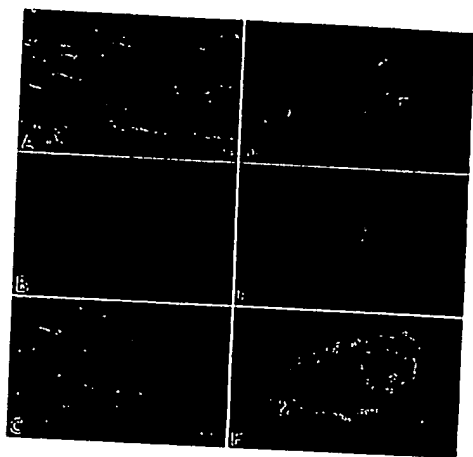


Fig. 3. Markers of differentiating cardiac cells. (A–F) Labeling of M by nestin (A, yellow), desmin (B, red), and connexin 43 (C, green); red fluorescence = cardiac myosin (A and C). (D and E) Yellow-green fluorescence reflects labeling of EC by flk-1 (arrows, D) and VE-cadherin (arrows, E); red fluorescence = factor VIII in EC (D and E). (F) Green fluorescence labeling of SMC cytoplasm by flk-1; endothelial lining is also labeled by flk-1; red fluorescence = α -smooth muscle actin; blue fluorescence = propidium iodide (PI) labeling of nuclei. (A and E, $\times 1,200$; B and F $\times 800$; C, $\times 1,400$; D, $\times 1,800$.)

of differentiation of M (10, 24, 25): nestin, desmin, α -sarcomeric actin, cardiac myosin, and connexin 43. Nestin was recognized in individual cells scattered across the forming band (Fig. 3A). With this exception, all other M expressed desmin (Fig. 3B), α -sarcomeric actin, cardiac myosin, and connexin 43 (Fig. 3C). Three transcription factors implicated in the activation of the promoter of several cardiac muscle structural genes were examined (10, 22, 23): Csx/Nkx2.5, GATA-4, and MEF2 (Fig. 7A–C, which is published as supplemental data on the PNAS web site). Single cells positive for flk-1 and VE-cadherin (26, 27), two EC markers, were present in the repairing tissue (Fig. 3D and E); flk-1 was detected in SMC isolated or within the arteriolar wall (Fig. 3F). This tyrosine kinase receptor promotes migration of SMC during angiogenesis (28). Therefore, repair of the infarcted heart involved growth and differentiation of all cardiac cell populations, resulting in *de novo* myocardium.

Myocardial Repair Improved Anatomical Remodeling and Ventricular Function. Myocardial regeneration attenuated cavity dilation and mural thinning during the evolution of the infarcted heart *in vivo*. Echocardiographically, LV end-systolic (LVESD) and end-diastolic diameters (LVEDD) increased more in nontreated than in cytokine-treated mice at 9, 16, and 26 days after infarction (Fig. 8A and B, which is published as supplemental data on the PNAS web site). Infarction prevented the evaluation of anterior wall systolic thickness (AWST) and anterior wall diastolic thickness (AWDT). When measurable, posterior wall thickness in systole (PWST) and diastole (PWDT) was greater in treated mice (Fig. 8C and D). Anatomically, the wall bordering and remote from infarction was 26% and 22% thicker in cytokine-injected mice (Fig. 8E). BMC-induced repair resulted in a 42% higher wall thickness to chamber radius ratio (Fig. 4A). Additionally, tissue regeneration decreased the expansion in cavity diameter (–14%), longitudinal axis (–5%; Fig. 8F and G), and chamber volume (–26%; Fig. 4B). Importantly, ventricular mass to chamber volume ratio was 36% higher in treated animals (Fig. 4C). Therefore, BMC mobilization that led to proliferation and differentiation of a new population of M and

vascular structures attenuated the anatomical variables which define cardiac decompensation.

Measurements of EF during the evolution of infarction and hemodynamics at the time of death showed that repair improved ventricular performance. EF was 48, 62, and 114% higher in treated than in nontreated mice at 9, 16, and 26 days after coronary occlusion, respectively (Fig. 4D). In mice exposed to cytokines, contractile function developed with time in the infarcted region of the wall (Fig. 4E–M; Fig. 8H–P). Conversely, LV end-diastolic pressure (LVEDP) increased 76% more in nontreated mice. The changes in LV systolic pressure (not shown), developed pressure (LVDP), and + and – dp/dt were also more severe in the absence of cytokine treatment (Fig. 9A–D, which is published as supplemental data on the PNAS web site). Additionally, the increase in diastolic stress in the zone bordering and remote from infarction was 69–73% lower in cytokine-treated mice (Fig. 4N). Therefore, cytokine-mediated infarct repair restored a noticeable level of contraction in the regenerating myocardium, decreasing diastolic wall stress and increasing ventricular performance.

Discussion

On the basis of the results presented above, we conclude that cytokine administration, with the consequent mobilization of BMC into the circulation and, presumably, their translocation to the infarcted portion of the heart, led to a significant magnitude of myocardial repair. Tissue regeneration comprised parenchymal cells and vascular structures. This anatomical restoration was accompanied by a dramatic reduction in post-MI mortality and a remarkable recovery in ventricular performance. Such a high degree of anatomical and functional improvement was accomplished in 100% of the treated animals by using a noninvasive procedure. The importance of these observations for the potential treatment of ischemic heart disease in humans is apparent.

The ability of exogenous BMC to home to the damaged area of the myocardium and differentiate into cells of the cardiogenic lineage, including coronary arterioles and capillaries, was shown previously by local transplantation of Lin[–] c-kit^{POS} cells into the border zone of an acute infarct (10). However, thoracic surgery and injection of foreign cells were required. Because of the complexity of the protocol, the rate of success with this invasive approach was only 40%. Additionally, this procedure required the availability of syngeneic donors as the source of the transplanted cells. In contrast, the methodology described here succeeded in all cases, eliminated the mortality and morbidity of thoracic surgery and, most importantly, obviated the use of foreign cells with the risk of transmission of infectious agents and the activation of an immunological reaction. Obviously, the generation of true myocardium and coronary vessels is superior to the use of skeletal myoblasts as a replacement for dead cardiac tissue (4, 5, 29). Although skeletal myoblasts survive and differentiate into skeletal muscle when injected into the myocardium, they never become electrically coupled with the rest of the heart because they do not express connexin 43 (4, 5, 29). Moreover, the diastolic properties of skeletal muscle cells are different from those of cardiomyocytes. Similarly, the formation of vessels only severely limits the possibility of complete functional repair after a segmental loss of ventricular mass (30).

Connexin 43 was clearly detectable in the newly formed M derived from BMC at 9 days after implantation (10) and it acquired a more mature pattern of distribution at 27 days. Consistent with the identification of contractile activity in the repairing myocardium, the expression of connexin 43 suggests that operative gap junctions were developed between M. To our knowledge, with the exception of this and our previous report using BMC, none of the published attempts to repair cardiac tissue post-MI have resulted in the production of functional, healthy myocardium. Although not investigated here, this con-

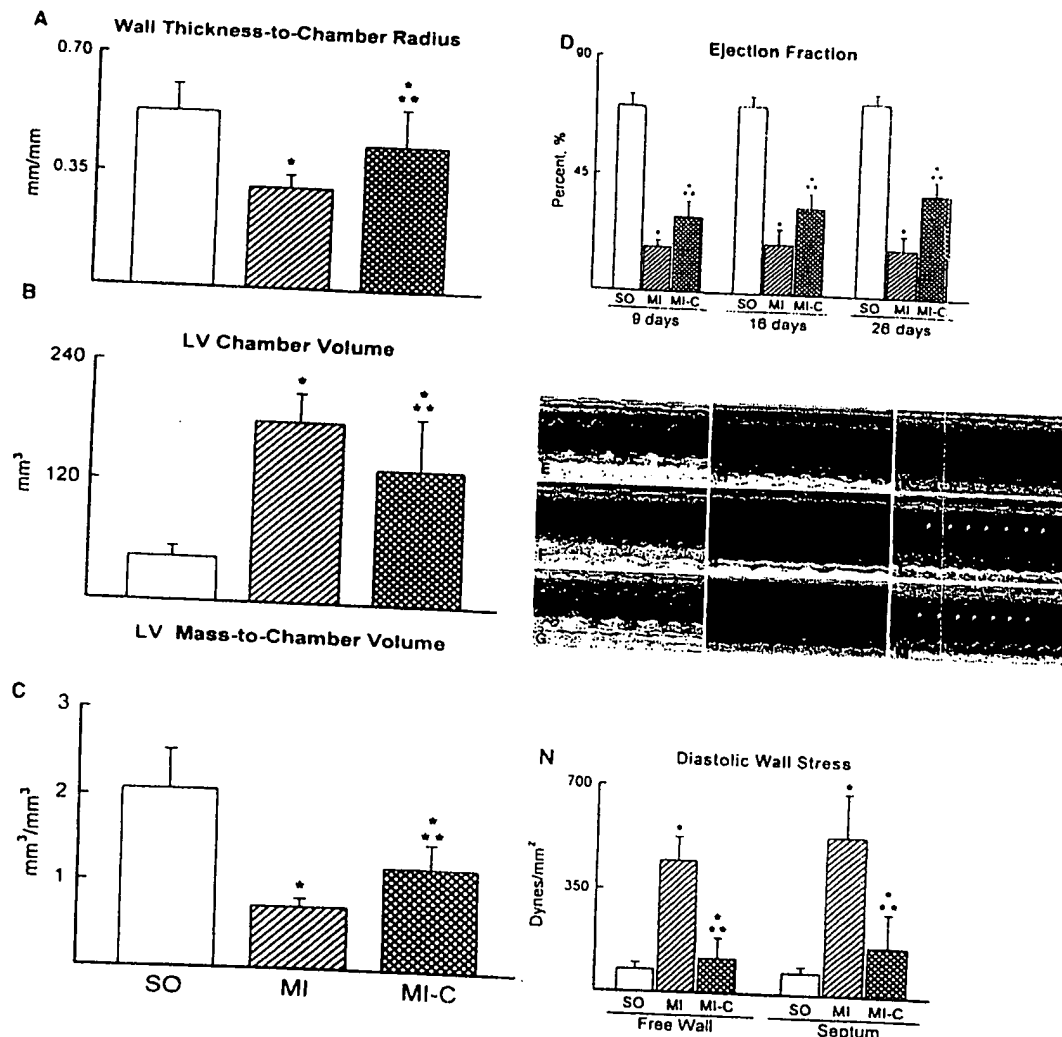


Fig. 4. MI, cardiac anatomy, and function. (A–C) LV dimensions at time of death, 27 days after surgery; SO ($n = 9$), nontreated infarcted (MI, $n = 9$), and cytokine-treated infarcted (MI-C, $n = 10$). (D) EF by echocardiography (SO, $n = 9$; MI, $n = 9$; and MI-C, $n = 9$). (E–M) M-mode echocardiograms of SO (E–G), MI (H–J), and MI-C (K–M); newly formed contracting myocardium (arrows). Detailed echocardiograms are shown in Fig. 8. (N) Wall stress, SO ($n = 9$); MI ($n = 8$); and MI-C ($n = 9$). Results are mean \pm SD. * and **, $P < 0.05$ vs. SO and MI, respectively.

tion implies that extracellular matrix supporting parenchymal cells and coronary vessels had to be formed. Additionally, scar formation was minimal in the treated animals but this does not exclude the notion that groups of myofibroblasts were present at the edges of the regenerating tissue.

The long-term unfavorable outcome of the infarcted heart is directly related to the initial infarct size that determines the degree of impaired pump function and the magnitude of dilation and wall thinning (31, 32). The changes in cardiac anatomy acutely after infarction, in combination with elevated filling pressure and decreased systolic pressure, induce large increases in diastolic stress and modest increases in systolic stress (18, 31). These structural-functional modifications promote chronic remodeling and the evolution of the myopathy to terminal failure (19, 31). Formation of new myocardium within the infarct attenuated the anatomical alterations, led to chronic increases in

EF, and reduced the abnormalities in cavity pressure, contractility, and loading. Longer intervals after homing of primitive BMC may result in complete repair of the infarcted heart.

Despite the success of the protocol used here, there are questions that we are currently addressing. Administration of SCF and G-CSF mobilizes pluripotent $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells from the bone marrow to the peripheral blood (13). The number of circulating $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells increased 250-fold. Donor BMC injected intravenously home to injured organs including liver (8) and skeletal muscle (33). Because of previous results with $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells (10), we propose that $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells are responsible for cardiac repair. Our results do not provide unambiguous information about the origin of the cells reconstituting the myocardium. It could be argued that cytokine treatment mobilized bone marrow stem cells and resident cardiac stem cells, which together participated in tissue regeneration. We have begun gene-marking studies in an effort to document

the plasticity of adult Lin⁺ c-kit^{POS} bone marrow cells in myocardial repair. This issue could also be addressed by treatment of irradiated animals with cytokines or by transfusing infarcted animals with BMC from syngeneic sex-mismatched donors.

The efficacy of cytokine treatment starting 5 days before MI followed by 3 more days postcoronary occlusion raises the question of the most effective therapeutic window. Additionally, it is not clear whether tissue repair is a result of the homing of BMC to the lesion or whether BMC, once mobilized, nest randomly throughout the organism and only those in the damaged myocardium rapidly proliferate and transdifferentiate. The former possibility is more attractive and supported by the rapid induction of SCF in a number of tissues, including the myocardium (34), in response to injury (34–37). SCF could be responsible for migration, accumulation, and multiplication of primitive BMC in the infarcted zone where they acquire the heart muscle phenotype reaching functional competence.

In conclusion, BMC injected or mobilized to the damaged myocardium behave as cardiac stem cells, giving rise to M, endothelial cells, and smooth muscle cells. Such behavior is no longer surprising given the remarkable plasticity of adult bone marrow stem cells (8–12, 30). New evidence offers clues as to some of the biochemical pathways implicated in this transdifferentiation. The interplay between the signal transduction pathways of bone morphogenetic proteins and the *Wnt* family of genes is responsible for the expression of lineage-determining

genes that condition whether a mesodermal precursor cell becomes a blood cell or a cardiac cell (38, 39). During development, the differentiation into a M or a hemopoietic cell is an opposing and mutually exclusive choice. This choice, established by the nature of the cell, seems to be influenced by cues from the environment where the cell resides. Such environmental cues have proven to be difficult to elucidate in the maturing embryo. Surprisingly, as shown here and previously (10, 13), adult BMC remain open to both developmental pathways and readily reprogram themselves in response to the habitat. The absence of hematopoietic islands in the regenerating myocardium as a result of BMC localization is further testimony of the responsiveness of these cells to environmental factors. Thus, this system offers a favorable experimental setting to uncover the biology of this intriguing and potentially clinically useful transdifferentiation.

We believe that the approach presented here might bring myocardial regeneration closer to clinical reality and might also offer the opportunity to uncover the molecular mechanisms involved.

We thank Dr. Seigo Izumo (Beth Israel Deaconess Medical Center, Boston) for providing us with the Csx/Nkx2.5 Ab. The mAb Rat-401 (anti-nestin) developed by S. Hockfield was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development, and maintained by the University of Iowa, Iowa City. This work was supported by National Institutes of Health Grants HL-38132, HL-39902, HL-43023, AG-15756, AG-17042, HL-66923, and HL-65577.

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roughly 300 animals, and given a choice between an optimal concentration of benzaldehyde (1:200 dilution in ethanol) and a lower concentration of diacetyl (1:10,000 dilution in ethanol) in the presence of a uniform field of butanone (1.2 μ l per 10-ml plate). Under these conditions more than 95% of wild-type animals prefer benzaldehyde. Animals that accumulated at the diacetyl source were removed and retested under the same conditions to repeat the enrichment. Animals that preferred diacetyl three times were isolated, and their F₁ broods were given a choice between benzaldehyde and diacetyl in the absence of a uniform concentration of butanone. Mutants that could chemotax to benzaldehyde under these conditions were saved. Twenty-seven mutants exhibited discrimination defects that could also be replicated without the diacetyl counterattractant. Mutants were backcrossed twice to wild-type animals.

Genetic mapping of *ky542*

We mapped *ky542* to chromosome II by observing segregation of the discrimination phenotype away from the dominant marker *sqr-1(scl)* (7/7 isolates). Subsequent mapping was performed by following segregation of the discrimination phenotype with single-nucleotide polymorphisms (SNPs) between the wild-type N2 and CB4856 strains. F₁ progeny of *ky542* × CB4856 crosses were isolated, and populations were generated from each isolate. Each population was tested for butanone/benzaldehyde discrimination. Populations that were homozygous mutant and those that were homozygous wild type were retained, whereas populations that appeared to be heterozygous were discarded. We isolated DNA from each population, and scored SNPs by polymerase chain reaction amplification followed by restriction-enzyme digestion. Using 33 populations, we found that *ky542* mapped between SNPs located on cosmid C01F1 (chromosome II, position -4.5) and cosmid C34F1 (chromosome II, position -2.5).

Laser ablations

AWC neurons were ablated in a wild-type strain that contained an integrated *str-2::GFP* reporter (*ky5140*) at the L1 or L2 larval stage¹². The AWC neuron was identified by its characteristic position or by the use of the *str-2::GFP* marker, and then laser irradiated. Ablation was confirmed for AWC^{ON}-ablated animals by looking for *str-2::GFP* expression after all assays had been performed. Single-animal assays were performed on gravid adults as early as the second day after ablation and as late as the fourth day. We assayed the same animals on two or three consecutive days. As many as three consecutive olfactory assays were performed in a single day. For discrimination assays, in which animals were challenged with the same attractant in the presence and absence of saturating odour, animals were allowed to recover between tests for 1 h on a fresh plate with no odours. The order of the assays was randomized on different days. Single-animal assay plates were poured 1 day before the assays and allowed to air dry for 1 h before the assays.

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Bone marrow cells regenerate infarcted myocardium

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Myocardial infarction leads to loss of tissue and impairment of cardiac performance. The remaining myocytes are unable to reconstitute the necrotic tissue, and the post-infarcted heart deteriorates with time¹. Injury to a target organ is sensed by distant stem cells, which migrate to the site of damage and undergo alternate stem cell differentiation^{2–5}; these events promote structural and functional repair^{6–8}. This high degree of stem cell plasticity prompted us to test whether dead myocardium could be restored by transplanting bone marrow cells in infarcted mice. We sorted lineage-negative (Lin[−]) bone marrow cells from transgenic mice expressing enhanced green fluorescent protein⁹ by fluorescence-activated cell sorting on the basis of *c-kit* expression¹⁰. Shortly after coronary ligation, Lin[−] *c-kit*^{POS} cells were injected in the contracting wall bordering the infarct. Here we report that newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplanting the bone marrow cells. The developing tissue comprised proliferating myocytes and vascular structures. Our studies indicate that locally delivered bone marrow cells can generate *de novo* myocardium, ameliorating the outcome of coronary artery disease.

Injection of male Lin[−] *c-kit*^{POS} bone marrow cells (see Supplementary Information) in the peri-infarcted left ventricle of female mice resulted in myocardial regeneration. Repair was obtained in 12 out of 30 mice (40%). Failure to reconstitute infarcts was attributed to the difficulty of transplanting cells into tissue contracting at 600 beats per minute. However, an immunological reaction to the histocompatibility antigen on the Y chromosome of the donor bone marrow cells could account for the lack of repair in some of the female recipients. Closely packed myocytes occupied 68 ± 11% of the infarcted region and extended from the anterior to the posterior aspect of the ventricle (Fig. 1a–d). The fraction of endocardial and epicardial circumference delimiting the infarcted area¹¹ did not differ in untreated mice, 78 ± 18% (*n* = 8), or in mice treated with Lin[−] *c-kit*^{POS} cells, 75 ± 14% (*n* = 12), or Lin[−] *c-kit*^{NEG} cells, 75 ± 15% (*n* = 11). New myocytes were not found in mice injected with Lin[−] *c-kit*^{NEG} cells (Fig. 1e).

The origin of the cells in the forming myocardium was deter-

mined by the expression of enhanced green fluorescent protein (EGFP) (Fig. 2; see also Supplementary Information) and the presence of Y chromosome (Supplementary Information). EGFP was restricted to the cytoplasm, whereas Y chromosome was restricted to the nuclei of new cardiac cells. EGFP and Y chromosome were not detected in the surviving portion of the ventricle. EGFP expression was combined with the labelling of proteins specific for myocytes, endothelial cells and smooth muscle cells. This allowed us to identify each cardiac cell type, and to recognize endothelial and smooth muscle cells organized in coronary vessels (Fig. 3a–c; see also Supplementary Information). The percentage of new myocytes, endothelial cells and smooth muscle cells expressing EGFP was $53 \pm 9\%$ ($n = 7$), $44 \pm 6\%$ ($n = 7$) and $49 \pm 7\%$ ($n = 7$), respectively. These values were consistent with the fraction of transplanted $\text{Lin}^{-}\text{c-kit}^{\text{POS}}$ bone marrow cells that expressed EGFP, $44 \pm 10\%$ ($n = 6$). An average $54 \pm 8\%$ ($n = 6$) of myocytes, endothelial cells and smooth muscle cells expressed EGFP in the heart of donor transgenic mice.

To confirm that newly formed myocytes represented maturing

cells aiming at functional competence, we examined expression of the myocyte enhancer factor 2 (MEF2), the cardiac specific transcription factor GATA-4 and the early marker of myocyte development *Csx/Nkx2.5*. In the heart, MEF2 proteins are recruited by GATA-4 to activate synergistically the promoters of several cardiac genes, such as myosin light chain, troponin T, troponin I, α -myosin heavy chain, desmin, atrial natriuretic factor and α -actinin^{12,13}. *Csx/Nkx2.5* is a transcription factor restricted to the initial phases of

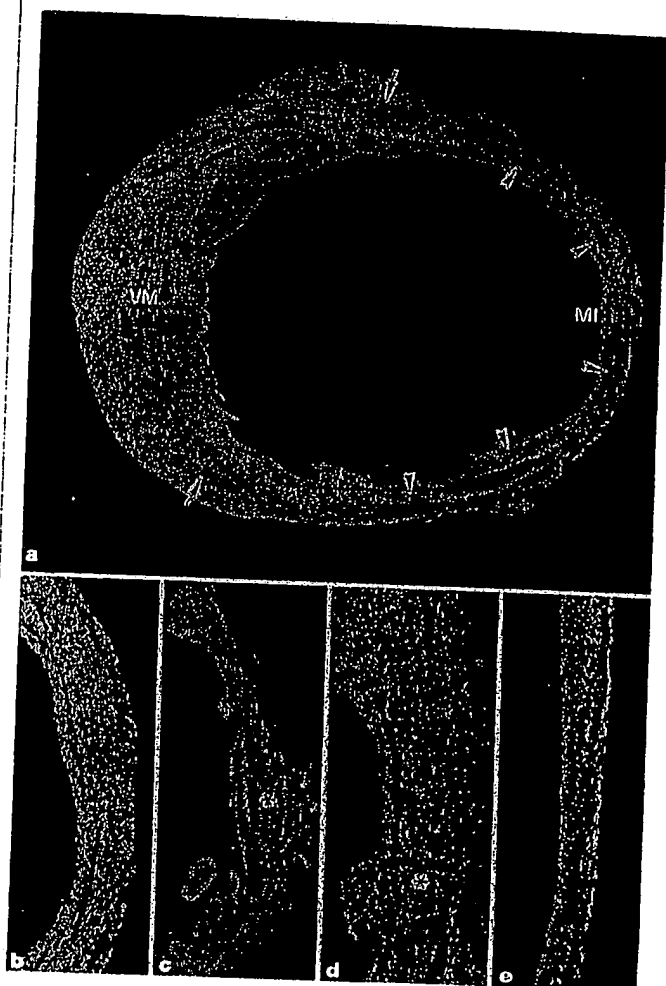


Figure 1 Bone marrow cells and myocardial regeneration. **a**, Myocardial infarct (MI) injected with $\text{Lin}^{-}\text{c-kit}^{\text{POS}}$ cells from bone marrow (arrows). Arrowheads indicate regenerating myocardium; VM, viable myocardium. **b**, Same MI at higher magnification. **c**, **d**, Low and high magnifications of MI injected with $\text{Lin}^{-}\text{c-kit}^{\text{POS}}$ cells. **e**, MI injected with $\text{Lin}^{-}\text{c-kit}^{\text{POS}}$ cells; only healing is apparent. Asterisk indicates necrotic myocytes. Red, cardiac myosin; green, propidium iodide labelling of nuclei. Original magnification, $\times 12$ (**a**); $\times 25$ (**c**); $\times 50$ (**b**, **d**, **e**).

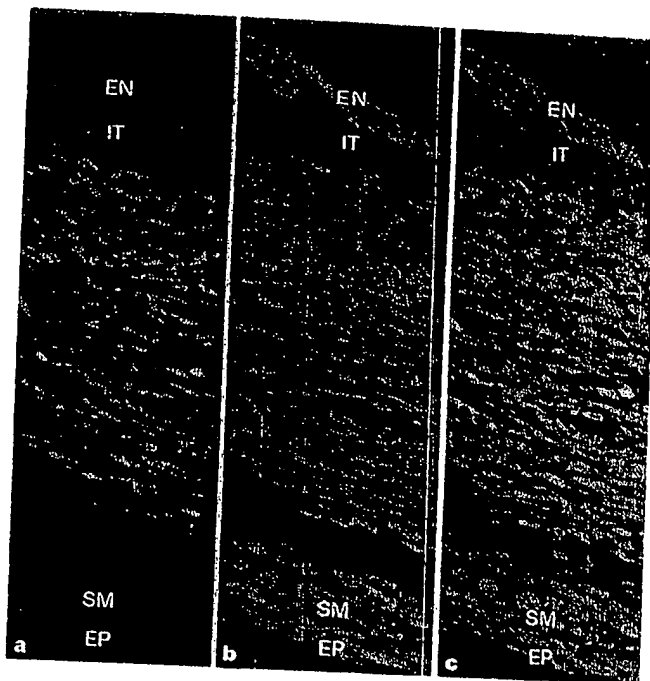


Figure 2 Myocardial infarct injected with $\text{Lin}^{-}\text{c-kit}^{\text{POS}}$ cells; myocardium is regenerating from endocardium (EN) to epicardium (EP). **a**, EGFP (green); **b**, cardiac myosin (red); **c**, combination of EGFP and myosin (red–green), and propidium iodide-stained nuclei (blue). Infarcted tissue (IT) can be seen in the subendocardium, spared myocytes (SM) can be seen in the subepicardium. Original magnification, $\times 250$ (**a–c**).

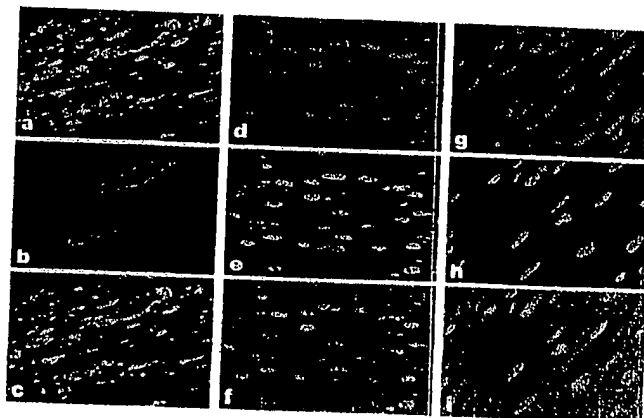


Figure 3 Regenerating myocardium in myocardial infarct injected with $\text{Lin}^{-}\text{c-kit}^{\text{POS}}$ cells. **a**, EGFP (green); **b**, smooth muscle α -actin in arterioles (red); **c**, combination of EGFP and smooth muscle α -actin (yellow–red), and propidium iodide (PI)-stained nuclei (blue). **d–i**, MEF2 and *Csx/Nkx2.5* in cardiac myosin-positive cells. **d**, **g**, PI-stained nuclei (blue); **e**, **h**, MEF2 and *Csx/Nkx2.5* labelling (green); **f**, **i**, cardiac myosin (red), and combination of MEF2 or *Csx/Nkx2.5* with PI (bright fluorescence in nuclei). Original magnification, $\times 300$ (**a–i**).

myocyte differentiation¹². In the reconstituting heart, all nuclei of cells labelled with cardiac myosin expressed MEF2 (Fig. 3d–f) and GATA-4 (Supplementary Information), but only $40 \pm 9\%$ expressed Csx/Nkx2.5 (Fig. 3g–i).

To characterize further the properties of these myocytes, we determined the expression of connexin 43. This protein is responsible for intercellular connections and electrical coupling through the generation of plasma-membrane channels between myocytes^{14,15}; connexin 43 was apparent in the cell cytoplasm and at the surface of closely aligned differentiating cells (Fig. 4). These results were consistent with the expected functional competence of the heart muscle phenotype. In addition, myocytes at various stages of maturation were detected within the same and different bands (Fig. 5).

Ki67 is expressed in cycling cells in G1, S, G2 and early mitosis¹⁶, providing a quantitative estimate of the fraction of cells in the cell cycle at the time of observation. 5-Bromodeoxyuridine (BrdU) labelling identifies nuclei in S phase^{16,17}; therefore, we injected BrdU for 4–5 days to assess cumulative cell division during active growth (Supplementary Information). Proliferation of myocytes was 93% ($P < 0.001$) and 60% ($P < 0.001$) higher than that of endothelial cells, and 225% ($P < 0.001$) and 176% ($P < 0.001$) higher than that of smooth muscle cells, when measured by BrdU and Ki67, respectively (BrdU: myocytes $36 \pm 8\%$; endothelial cells $19 \pm 5\%$; smooth muscle cells $11 \pm 2\%$; Ki67: myocytes $19 \pm 3\%$; endothelial cells $12 \pm 3\%$; smooth muscle cells $7 \pm 2\%$; $n = 8$ in all cases). Dividing myocytes were small with partially aligned myofibrils, resembling late fetal/neonatal cells; 40–50% of the Ki67- or BrdU-positive cells expressed EGFP.

Cell differentiation caused a loss of *c-kit* surface receptors. We observed only two undifferentiated cells showing *c-kit* on the cell

membrane in the subendocardium of the infarcted wall. These *c-kit*-labelled cells were in proximity but not within the regenerating band. They expressed EGFP, confirming their origin from the transplant (Supplementary Information).

To determine whether developing myocytes derived from the Lin[−]*c-kit*^{POS} cells had an impact on function, we obtained haemodynamic parameters before death. Results from infarcted mice non-injected or injected with Lin[−]*c-kit*^{NEG} cells were combined. In comparison with sham-operated mice, the infarcted groups exhibited indices of cardiac failure (Fig. 6a). In mice treated with Lin[−]*c-kit*^{POS} cells, left ventricular (LV) end-diastolic pressure (LVEDP) was 36% lower, and developed pressure (LVDP), LV + dP/dt and LV − dP/dt were 32%, 40% and 41% higher, respectively.

Locally transplanted Lin[−]*c-kit*^{POS} bone marrow cells have a high capacity for cardiac tissue differentiation. Here, they led to the formation of new myocytes, endothelial cells and smooth muscle cells generating *de novo* myocardium, inclusive of coronary arteries, arterioles and capillaries. The partial repair of the infarcted heart implies that the transplanted cells responded to signals from the injured myocardium that promoted their migration, proliferation and differentiation within the necrotic area of the ventricular wall (Fig. 6b). These differentiating myocytes expressed nuclear and cytoplasmic proteins typical of cardiac tissue. The presence of connexin 43 points to cellular coupling and functional competence of the restored myocardium (Fig. 6b). With postnatal maturation, stem cell function was assumed previously to be restricted to cell lineages present in the organ from which they are derived. However, this limitation in stem cell differentiation potential has been challenged by studies showing that bone marrow and neural stem cells can produce many cell types^{4,5,18–20}. We report, for the first time, that a subpopulation of primitive bone marrow cells regenerate myocardium *in vivo*, replacing dead tissue.

Haematopoietic stem cells (HSCs), neural-crest-derived melanoblasts and primordial germ cells express *c-kit* on their cell membrane. These primitive cells migrate during fetal development,

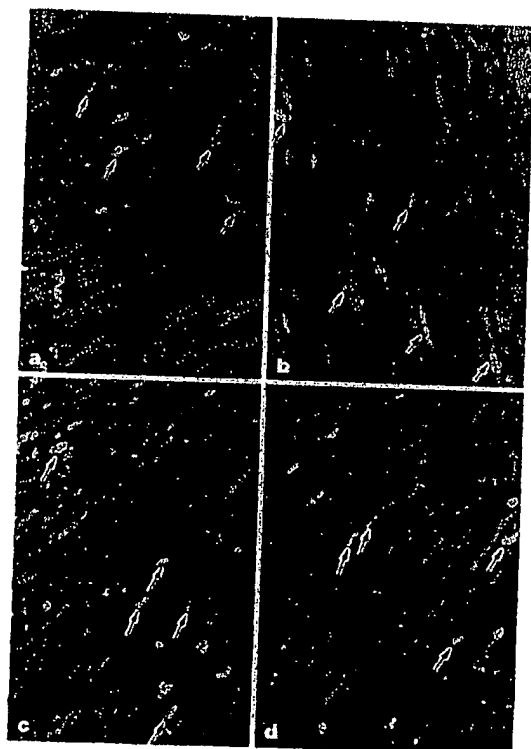


Figure 4 Myocardial repair and connexin 43. **a**, Border zone; **b–d**, regenerating myocardium. Shown are connexin 43 (yellow–green; arrows indicate contacts between myocytes) and α -sarcomeric actin (red), and PI-stained nuclei (blue). Original magnification, $\times 500$ (**a**), $\times 800$ (**b–d**).

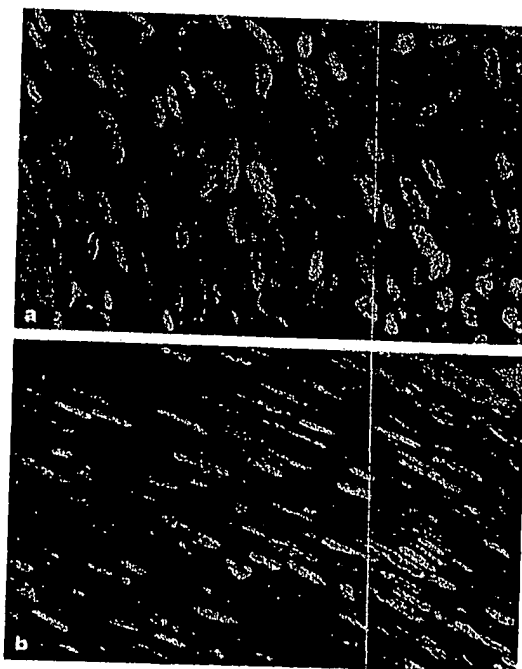


Figure 5 Myocardial infarcts injected with Lin[−]*c-kit*^{POS} cells: regenerating myocytes. Shown are cardiac myosin (red), and propidium-iodide-labelled nuclei (yellow–green). Original magnification, $\times 1,000$ (**a**); $\times 700$ (**b**).

homing to the yolk sac and liver. Both of these organs are positive for messenger RNA encoding stem cell factor (SCF), the ligand for *c-kit*¹¹. It is thought that membrane-bound SCF mediates the migration of HSC and other primitive cells to their target organs¹². The fetal and neonatal hearts are positive for SCF transcripts¹¹ and, although it is not clear whether adult heart cells generate SCF, the *c-kit*/SCF pathway might be the mechanism by which, in our conditions, transplanted *Lin*⁻*c-kit*^{POS} cells migrated from the site of injection to the infarcted myocardium.

When a stem cell divides, two daughter cells are formed; these may maintain stem cell properties or become differentiating cells²³ that multiply much more rapidly than stem cells²⁴. The *Lin*⁻*c-kit*^{POS} cells in these transplants produced the three main cell types of the heart: myocytes constituted the predominant and most active growth component of the regenerating myocardium; endothelial and smooth muscle cells were fast growing but were smaller fractions of the developing tissue. Our observations are difficult

to compare with those obtained in the cryo-injured rat heart after injecting cultured myocytes derived from mesenchymal bone marrow cells²⁵. Formation of myotubules *in vitro* was required for successful transplantation in that study²⁵, which contrasts with our results. Cryo-injury has no human counterpart. It constitutes an unusual damage with an intact coronary circulation. This may be why only a few endothelial cells were possibly linked to the original culture system²⁵ and smooth muscle cells were not detected. Also at variance with our data is the fact that there was no replacement of damaged myocardium with functioning tissue.

Coronary heart disease accounts for 50% of all cardiovascular deaths and nearly 40% of the incidence of heart failure. The current findings have provided compelling evidence that our approach has relevant implications for human disease. Locally delivered primitive bone marrow cells promoted successful treatment of large myocardial infarcts after the completion of ischaemic cell death. This therapeutic intervention reduced the infarcted area and improved cardiac haemodynamics. Infarct size is a major determinant of morbidity and mortality, as massive infarcts affecting 40% or more of the left ventricle in patients are associated with intractable cardiogenic shock or the rapid development of congestive heart failure¹. In the past, recovery of cardiac function has been fully dependent on the growth of the remaining non-infarcted portion of the ventricle. However, the hypertrophied infarcted heart undergoes progressive deterioration, leading to a dilated myopathy, terminal failure and death¹. Transplanted *Lin*⁻*c-kit*^{POS} bone marrow cells have the capability of regenerating acutely significant amounts of contracting myocardium. This new form of repair can improve the immediate and long-term outcome of ischaemic cardiomyopathy.

Methods

Lin⁻*c-kit*^{POS} cells

We collected bone marrow from the femurs and tibias of male transgenic mice expressing EGFP⁹. Cells were suspended in PBS containing 5% fetal calf serum (FCS) and incubated on ice with rat anti-mouse monoclonal antibodies specific for the following haematopoietic lineages: CD4 and CD8 (T lymphocytes), B-220 (B lymphocytes), Mac-1 (macrophages), GR-1 (granulocytes) (all Caltag Laboratories) and TER-119 (erythrocytes) (Pharmingen). Cells were then rinsed in PBS and incubated for 30 min with magnetic beads coated with goat anti-rat immunoglobulin (Polysciences). Lineage-positive cells were removed by a biomagnet and the 10% remaining lineage-negative (*Lin*⁻) cells were stained with ACK-4-biotin (anti-*c-kit* monoclonal antibody). Cells were rinsed in PBS, stained with streptavidin-conjugated phycoerythrin (SA-PE) (Caltag) and sorted by FACS using a FACSVantage instrument (Becton Dickinson). We excited EGFP and ACK-4-biotin-SA-PE at a wavelength of 488 nm. The *Lin*⁻ cells were sorted as *c-kit*^{POS} (*c-kit*^{POS}) and *c-kit*^{NEG} (*c-kit*^{NEG}) with a 1–2 log difference in staining intensity. The *c-kit*^{POS} cells were suspended at a concentration of 3×10^4 to 2×10^5 cells in 5 μ l of PBS, and the *c-kit*^{NEG} cells were suspended at a concentration of 5×10^4 to 5×10^5 cells in 5 μ l of PBS¹⁰.

Myocardial infarction

Myocardial infarction was induced in female C57BL/6 mice at 2 months of age as described¹⁰; 3–5 h after infarction, the thorax was re-opened and 2.5 μ l PBS containing *Lin*⁻*c-kit*^{POS} cells were injected in the anterior and posterior aspects of the viable myocardium bordering the infarct. Infarcted mice that were not injected or injected with *Lin*⁻*c-kit*^{NEG} cells and sham-operated mice were used as controls. All animals were killed 9 \pm 2 days after surgery. Protocols were approved by an institutional review board.

Ventricular function

Mice were anaesthetized with chloral hydrate (400 mg per kg (body weight)), intraperitoneally (i.p.), and the right carotid artery was cannulated with a microtip pressure transducer (model SPR-671; Millar) for the measurements of LV pressures, and LV + and LV - dP/dt in the closed-chest preparation. The abdominal aorta was cannulated, the heart was arrested in diastole, and the myocardium was perfused retrogradely with 10% buffered formalin^{10,14}. Three tissue sections, from the base to the apex of the left ventricle, were stained with haematoxylin and eosin. At 9 \pm 2 days after coronary occlusion, the infarcted portion of the ventricle was easily identifiable grossly and histologically (see Fig. 1a). The lengths of the endocardial and epicardial surfaces delimiting the infarcted region, and the endocardium and epicardium of the entire left ventricle, were measured in each section. Subsequently, their quotients were computed to yield the average infarct size in each case. This was accomplished at $\times 4$ magnification with an image analyser connected to a microscope¹¹.

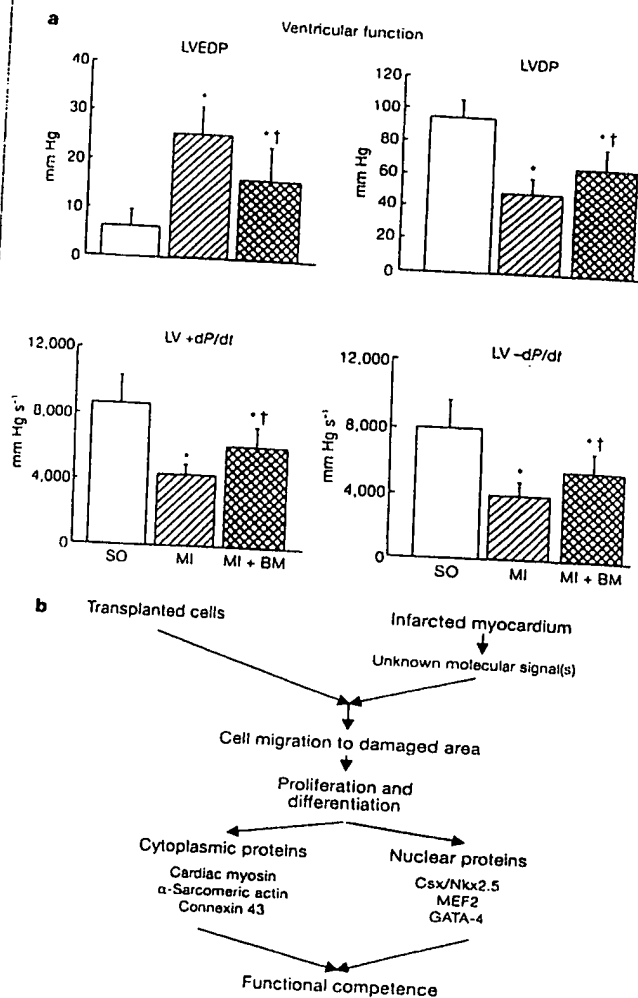


Figure 6 Postulated mechanism of myocardial regeneration and its effect on ventricular function. **a**, Effects of myocardial infarction (MI) on left ventricular end-diastolic pressure (LVEDP), developed pressure (LVDP), LV + dP/dt (rate of pressure rise) and LV - dP/dt (rate of pressure decay). Results are from sham-operated mice (SO, $n = 11$), mice non-injected with *Lin*⁻*c-kit*^{POS} cells (MI; $n = 5$ injected with *Lin*⁻*c-kit*^{NEG} cells; $n = 6$ non-injected), and mice injected with *Lin*⁻*c-kit*^{POS} cells (MI+BM, $n = 9$). Values are mean \pm s.d. * $P < 0.05$ versus SO; † $P < 0.05$ versus MI. **b**, Proposed scheme for *Lin*⁻*c-kit*^{POS} cell differentiation in cardiac muscle and functional implications.

Cell proliferation and EGFP detection

To establish whether Lin⁺c-kit^{POS} cells resulted in myocardial regeneration, we administered BrdU (50 mg per kg (body weight), i.p.) to the animals daily for 4–5 consecutive days before death. Sections were incubated with anti-BrdU antibody, and BrdU labelling of cardiac cells was measured¹⁷. Moreover, expression of Ki67 in nuclei was evaluated by treating samples with a rabbit polyclonal anti-mouse Ki67 antibody (Dako). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was used as secondary antibody. EGFP was detected with a rabbit polyclonal anti-GFP (Molecular Probes). Myocytes were recognized with a mouse monoclonal anti-cardiac myosin heavy chain (MAB 1552; Chemicon) or a mouse monoclonal anti-sarcomeric α -actin (clone SC5; Sigma). Endothelial cells with a rabbit polyclonal anti-human factor VIII (Sigma) and smooth muscle cells with a mouse monoclonal anti-smooth-muscle α -actin (clone 1A4; Sigma). Nuclei were stained with propidium iodide, 10 μ g ml⁻¹ (refs 27, 28). We determined the percentages of myocyte (M), endothelial cell (EC) and smooth muscle cell (SMC) nuclei labelled by BrdU and Ki67 by confocal microscopy. This was accomplished by dividing the number of nuclei labelled by the total number of nuclei examined. Numbers of nuclei sampled in each cell population were as follows. BrdU labelling: M, 2,908; EC, 2,153; SMC, 4,877. Ki67 labelling: M, 3,771; EC, 4,051; SMC, 4,752. Numbers of cells counted for EGFP labelling: M, 3,278; EC, 2,056; SMC, 1,274. We determined the percentage of myocytes in the regenerating myocardium by delineating the area occupied by cardiac-myosin-stained cells and dividing this by the total area represented by the infarcted region in each case.

Y chromosome

For the fluorescence *in situ* hybridization assay, we exposed sections to a denaturing solution containing 70% formamide. After dehydration with ethanol, sections were hybridized with the DNA probe CEP Y (sarcosine III) Spectrum Green (Vysis) for 3 h (ref. 29). Nuclei were stained with propidium iodide.

Transcription factors and connexin 43

Sections were incubated with rabbit polyclonal anti-MEF2 (C-21; Santa Cruz), rabbit polyclonal anti-GATA-4 (H-112; Santa Cruz), rabbit polyclonal anti-Csx/Nkx2.5 (obtained from Dr S. Izumo) and rabbit polyclonal anti-connexin 43 (Sigma). We used FITC-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody²⁰.

Statistical analysis

Results are presented as means \pm s.d. Significance between two measurements was determined by Student's *t*-test, and in multiple comparisons was evaluated by the Bonferroni method. Values of $P < 0.05$ were considered significant.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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CaT1 manifests the pore properties of the calcium-release-activated calcium channel

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The calcium-release-activated Ca^{2+} channel, I_{CRAC}^{1-3} , is a highly Ca^{2+} -selective ion channel that is activated on depletion of either intracellular Ca^{2+} levels or intracellular Ca^{2+} stores. The unique gating of I_{CRAC} has made it a favourite target of investigation for new signal transduction mechanisms; however, without molecular identification of the channel protein, such studies have been inconclusive. Here we show that the protein CaT1 (ref. 4), which has six membrane-spanning domains, exhibits the unique biophysical properties of I_{CRAC} when expressed in mammalian cells. Like I_{CRAC} , expressed CaT1 protein is Ca^{2+} selective, activated by a reduction in intracellular Ca^{2+} concentration, and inactivated by higher intracellular concentrations of Ca^{2+} . The channel is indistinguishable from I_{CRAC} in the following features: sequence of selectivity to divalent cations; an anomalous mole fraction effect; whole-cell current kinetics; block by lanthanum; loss of selectivity in the absence of divalent cations; and single-channel conductance to Na^{+} in divalent-ion-free conditions. CaT1 is activated by both passive and active depletion of calcium stores. We propose that CaT1 comprises all or part of the I_{CRAC} pore.

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EVIDENCE APPENDIX

ITEM NO. 6

**Strauer et al. publication in Circulation entitled, “Repair of
Infarcted Myocardium by Autologous Intracoronary
Mononuclear Bone Marrow Cell Transplanattion in Humans”
cited by Applicant as Exhibit E in Declaration of Dr. Richard
Heuser filed June 17, 2003
(in connection with concurrently-filed Amendment)**

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

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Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12 \pm 7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.^{1,2} Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.³

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.⁴⁻⁷ Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.⁸⁻¹⁰ Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.¹¹⁻¹⁴ However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.¹⁵ Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.¹⁶ However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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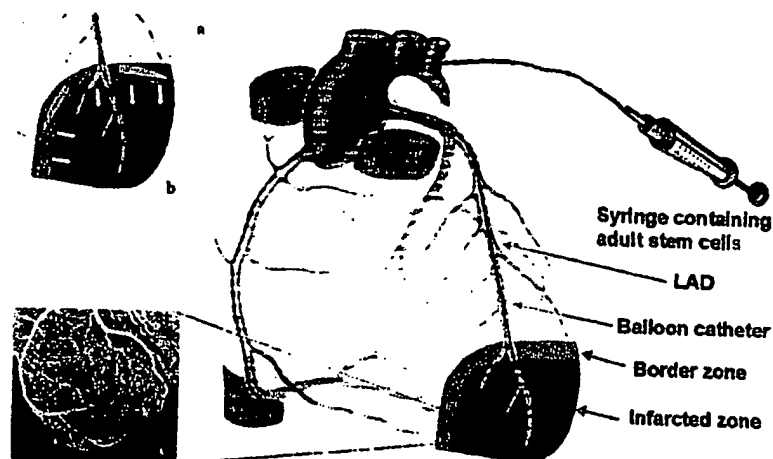


Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. a, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. b, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. c, A supply of blood flow exists within the infarcted zone.³⁵ The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.¹⁷ To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery ($n=4$), left circumflex coronary artery ($n=3$), or right coronary artery ($n=13$). Mean duration of infarct pain was 12 ± 10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty ($n=20$) and subsequent stent implantation ($n=19$). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β -blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (± 2) days after acute coronary angiography, bone marrow (~ 40 mL) was aspirated under local anesthesia from ilium of cell therapy patients ($n=10$). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O . For overnight

cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was $93 \pm 3\%$. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7 ; this consisted of $0.65 \pm 0.4\%$ AC133-positive cells and $2.1 \pm 0.28\%$ CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.¹⁸

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.¹⁹ All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2
Mononuclear bone marrow cells, n (×10 ³)	2.8±2.2

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ²⁰¹thallium scintigraphy. The contractility index $P_{\text{max}}/\text{ESV}$ was calculated by dividing LV systolic pressure (P_{max}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, $P=0.005$). It was also significantly smaller compared with the standard therapy group after 3 months ($P=0.04$). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, $P=NS$). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², $P=0.016$, assessed by ²⁰¹thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

- (1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², $P=0.010$) and ejection fraction (from 51±14 to 53±13%, $P=NS$).
- (2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, $P=NS$) and end-systolic volume (from 82±26 to 67±21 mL, $P=0.011$). Radionuclide ventriculography was used to acquire the data.
- (3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, $P=NS$, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, $P=0.005$).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dysknetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dysknetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility Indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow-derived cells in experimental infarction.^{11–14,18,20–23} Moreover, trans-endothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.^{3,12,24–26} Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.^{15,16} Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.²⁷ Adult, mononuclear BMCs contain such stem and progenitor cells ($\leq 1\%$), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

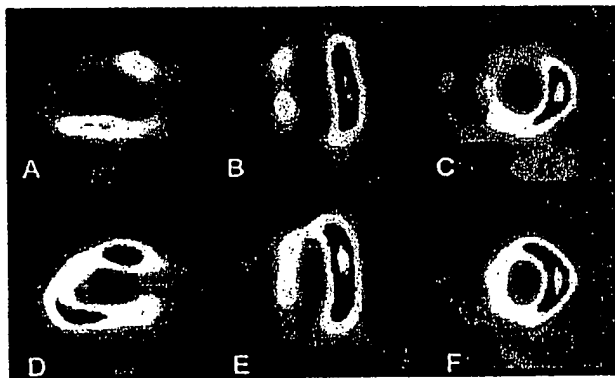


Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ²⁰¹thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m ²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility Indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P ₂₀₀ /ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
²⁰¹ Thallium scintigraphy, cm ²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells¹⁻³; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells²⁸; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.²⁹ Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.³⁰

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ~200 g) will flow per minute.^{31,32} This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).³¹ Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,³³ because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.³⁴ To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

- (1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.³⁵ Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

- (2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.³⁶ Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.
- (3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.³⁷⁻⁴⁰ Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility ($P_{1/2}$ /ESV and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.⁴¹ None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12 ± 10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.⁴¹ In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angiogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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EVIDENCE APPENDIX

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Heart Failure

Transendocardial Autologous Bone Marrow Mononuclear Cell Injection in Ischemic Heart Failure

Postmortem Anatomicopathologic and Immunohistochemical Findings

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► Abstract

Background—Cell-based therapies for treatment of ischemic heart disease are currently under investigation. We previously reported the results of a phase I trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease. The current report focuses on postmortem cardiac findings from one of the treated patients, who died 11 months after cell therapy.

Methods and Results—Anatomicopathologic, morphometric, and immunocytochemical findings from the anterolateral ventricular wall (with cell therapy) were compared with findings from the interventricular septum (normal perfusion and no cell therapy) and from the inferoposterior ventricular wall (extensive scar tissue and no cell therapy). No signs of adverse events were found in the cell-injected areas. Capillary density was significantly higher ($P<0.001$) in the anterolateral wall than in the previously infarcted tissue in the posterior wall. The prominent vasculature of the anterolateral wall was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (troponin, sarcomeric α -actinin, actinin), as well as the morphology of cardiomyocytes, and appeared to have migrated toward adjacent bundles of cardiomyocytes.

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Conclusions— Eleven months after treatment, morphological and immunocytochemical analysis of the sites of ABMM cell injection showed no abnormal cell growth or tissue lesions and suggested that an active process of angiogenesis was present in both the fibrotic cicatricial tissue and the adjacent cardiac muscle. Some of the pericytes had acquired the morphology of cardiomyocytes, suggesting long-term sequential regeneration of the cardiac vascular tree and muscle.

Key Words: angiogenesis • stem cells • heart failure • revascularization • ischemia

► Introduction

The role of cell-based therapy for the treatment of ischemic heart disease is currently under investigation. In view of the myocardium's limited capacity to regenerate spontaneously after an ischemic injury, the therapeutic use of exogenous progenitor cells has recently gained increasing interest. In vitro demonstration of functional cardiomyocyte differentiation from bone marrow–derived progenitor cells^{1,2} has prompted in vivo studies in animal models, and promising results have been obtained in the repair and regeneration of acute and chronic cardiac muscle lesions. Several types of progenitor cells have been used in experimental models, including bone marrow–derived endothelial and blood cell progenitors, as well as bone marrow mesenchymal progenitors.^{3–6}

In humans, similar attempts have been made with surgical, intracoronary, or transendocardial introduction of bone marrow–derived cells to improve cardiac lesions.^{7,8} Our group recently reported the results of the first phase I human trial of transendocardial injection of autologous bone marrow mononuclear (ABMM) cells in patients with end-stage ischemic heart disease.⁹ We observed a significant increase in perfusion, contractility of ischemic myocardial segments, and functional capacity of the cell-injection recipients. This report presents postmortem cardiac findings from one of these patients.

► Case Report

The patient was a 55-year-old man with ischemic cardiomyopathy and 2 previous myocardial infarctions (in 1985 and 2000). He began to have symptoms of congestive heart failure 2 years before study enrollment. One year before enrollment, the patient had an ischemic stroke with mild residual right hemiparesis

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and resultant episodes of chaotic tonic-clonic seizures. His risk factors for coronary artery disease included diabetes mellitus type II, hypertension, and hypercholesterolemia.

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The patient's functional capacity was evaluated at baseline by means of a ramp treadmill protocol¹⁰ with a peak maximal oxygen consumption ($\dot{V}O_{2\max}$) of $15.9 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ and a workload of 4.51 metabolic equivalents (METs). A baseline single-photon-emission computed tomography (SPECT) perfusion study showed a partially reversible perfusion defect in the anterolateral wall, a fixed perfusion defect (scar) in the inferior and posterior walls, and normal perfusion in the septal wall.

Cardiac catheterization revealed a left ventricular ejection fraction of 11%, a 70% ostial and an 85% middle stenosis of the left anterior descending (LAD) coronary artery, an 80% proximal lesion of the left circumflex (LCx) coronary artery, and total occlusion of the first obtuse marginal artery and right coronary artery. The distal segments of the LAD and LCx were diffusely diseased. Owing to the severity and extent of the patient's coronary disease, he was not considered a candidate for surgical or interventional procedures. At enrollment in our study, he was in New York Heart Association (NYHA) functional class III and Canadian Cardiovascular Society (CCS) angina class III. His serum C-reactive protein level, complete blood count, creatine kinase level, and troponin level were normal at baseline.

The patient received a total of 3×10^7 ABMM cells (the [Table](#)) that had been harvested 2 hours before the procedure. With the guidance of electromechanical mapping,^{11,12} the cells were injected transendocardially into the anterolateral wall of the left ventricle. No periprocedural complications were observed.

View this table: **Phenotype and Functional Characterization of 3×10^7 Cells Injected via a Transendocardial Route***
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Noninvasive follow-up evaluation was performed 2 and 6 months after cell therapy. Invasive follow-up evaluation, with cardiac catheterization, was performed at 4 months and revealed no change in coronary anatomy. Symptomatic and functional improvements were noted because the patient returned to NYHA and CCS class I. Holter monitoring showed no malignant ventricular arrhythmias, and signal-averaged ECG parameters remained stable. There was no change in the patient's medications after cell therapy. There was no change in the global ejection fraction or left

ventricular volume on echocardiography. The wall-motion index score (on 2-dimensional echocardiography) improved from 1.94 to 1.65 as contractility increased in 5 segments adjacent to the injected area. Myocardial perfusion, as assessed by SPECT, improved in the anterolateral wall. Mechanical data derived from SPECT showed improvements in regional ejection fraction, wall motion, and thickening. In addition, during ramp treadmill testing, the $\dot{V}O_2\text{max}$ increased from 15.8 to 25.2 mL · kg⁻¹ · min⁻¹, and the METs increased from 4.51 to 7.21 at 2 months. At 6-month follow-up testing, the $\dot{V}O_2\text{max}$ reached 31.6 mL · kg⁻¹ · min⁻¹, and the METs was 9.03.

From 6 to 11 months after the cell injection procedure, the patient's cardiovascular condition remained stable. At 11 months, however, he had a tonic-clonic seizure at home and was found in cardiopulmonary arrest by family members.

► **Methods**

After signed, informed consent was given by the family, an autopsy was performed, including morphological and immunocytochemical analysis of the heart. This report presents the anatomicopathologic findings about the infarcted areas of the anterolateral ventricular wall, which were the areas that had received bone marrow cell injections. The histological findings from this region were compared with findings from within the interventricular septum (which had normal perfusion in the central region and no cell therapy) and findings from the previously infarcted inferoposterior ventricular wall (which had extensive scarring and no cell therapy).

Immunocytochemical analysis of paraffin sections was performed with antibodies against factor VIII-related antigen (A0082, Dako), vimentin (M0725, Dako), smooth muscle α -actin (M0851, Dako), and CD34 and Ki-67 (NCL-L-End and NCL-Ki67MM1 respectively, Novocastra). Antibodies were reacted with Dako's EnVision+ System/HRP, with diaminobenzidine as a chromogen. Frozen sections were fixed, permeated with acetone, and incubated with antibodies for troponin T (T6277, Sigma), smooth muscle α -actin, sarcomeric actinin (A7811, Sigma), and desmin (D1033, Sigma). Antibodies were revealed with anti-mouse or anti-rabbit IgG, F(ab)₂ fragment, conjugated to fluorescein isothiocyanate (1814192 and 1238833, respectively, Boehringer-Mannheim), and counterstained with a 0.1% solution of Evans blue dye (Merck).

Capillary density was monitored by using computerized image analysis (Image-Pro Plus, MediaCybernetics) of randomly selected fields in sections stained with hematoxylin and reacted with antibody for factor VIII-related antigen (n=108) and randomly selected fields in sections reacted with antibodies for smooth muscle α -actin (n=96). Transverse sections of capillaries identified by staining

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for factor VIII and pericyte-containing capillaries identified by staining for smooth muscle α -actin were quantified separately. Results were expressed as the mean number of capillaries per square millimeter in the case of factor VIII-stained slides or the number of capillaries containing pericytes in α -smooth-muscle-actin-stained slides. Larger vessels identified by a continuous wall of smooth muscle actin-positive mural cells were excluded. Differences between the anterolateral, septal, and posterior walls were assessed with Kruskal-Wallis ANOVA and the Student-Newman-Keuls method for pairwise multiple comparison. Results were considered significant if P was <0.05 .

Evaluation of the capillary density inside the fibrotic areas within the cell-treated anterolateral wall versus the nontreated posterior wall was performed in 40 selected fields inside the fibrotic scars, excluding the regions containing cardiomyocytes. Microscope fields (at $\times 100$) of factor VIII-stained slides were digitized, and the number of transverse sections of capillaries per square millimeter of fibrotic zones was assessed. Differences between the treated infarcted zones and the nontreated fibrotic wall were assessed by the Mann-Whitney rank-sum test. Results were considered significant if P was <0.05 .

► Results

Anatomopathologic Findings

The heart weighed 765 g. There was severe arteriosclerosis with subocclusive calcified atheromata in all coronary arteries, calcification of the pulmonary artery, and moderate atheromatosis of the aorta. The heart cavities were dilated, with hypertrophic walls. There was no evidence of any acute injury or of lesions that could be related to cell injections. A generalized, homogeneous endocardial opacification, affecting all the cardiac internal surfaces, was identified on histological examination as diffuse fibroelastic hyperplasia of the endocardium. Minute focal and punctate scars were observed, mainly in the posterior and anterolateral walls.

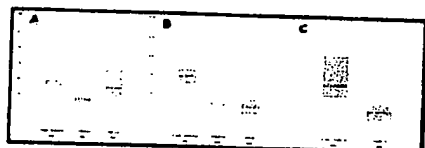
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The apical zone was thinned and fibrotic. The posterior and apical regions had dense, fibrotic, well-circumscribed scars that separated cardiomyocyte bundles. The septal wall exhibited focal scars interspersed with cardiac fibers in the regions adjacent to the anterior and posterior ventricular walls, but it was devoid of fibrosis in the central region.

The anterolateral ventricular wall that received cell injections had elongated, irregular, and parallel reddish areas throughout. In the same wall, in adjacent regions that did not receive injections, the density and morphology of the fibrotic scars were similar to those of the posterior wall, suggesting that no overt differences were present among the different infarcted areas before cell injections.

Morphometric Analysis

The capillary density was significantly higher in the areas of the anterolateral ventricular walls that received cell injections than in the previously infarcted posterior wall ($P < 0.0001$) (Figure 1 A). The median capillary density in the anterolateral wall was apparently similar to that in the septal wall. However, the broad dispersion of the septal wall data, which may have been due to fibrotic areas in regions close or adjacent to the ventricular walls, generated a statistically significant difference between these 2 groups.



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Figure 1. Number of capillaries per mm^2 in anterolateral, posterior, and septal walls of studied heart. A, Anti-factor VIII-associated antigen counterstained with hematoxylin. B, Anti-smooth muscle α -actin antigen counterstained with hematoxylin. C, Capillaries reacted with anti-factor VIII-associated antigen inside fibrotic areas only in anterolateral and posterior walls. (n=108 microscope fields for A; 96 microscope fields for B; and 40 microscopic fields for C.) Differences were statistically significant among all groups in pairwise comparisons ($P < 0.05$, Newman-Keuls method) for A and B. Differences were significantly different ($P < 0.05$) between anterolateral and posterior walls in Mann-Whitney rank-sum test for C.

The density of capillaries that contained smooth muscle α -actin-positive cells within their walls was also assessed (Figure 1 B). The number of such vessels was higher in the anterolateral wall than in the septal and posterior walls ($P < 0.0001$). Larger vessels identified by a continuous wall of smooth muscle α -actin-positive mural cells were not included in these analyses. The capillary density was significantly higher within fibrotic areas of the anterolateral wall than within fibrotic areas of the posterior wall ($P < 0.0001$) (Figure 1 C).

Histological Findings

The anterolateral wall showed irregular, pale regions of fibrotic tissue intercalated with dark regions of cardiac muscle arranged in roughly parallel, interspersed bands, perpendicular to the ventricular wall plane (Figure 2 A). No abnormal cell organization, growth, or differentiation or signs of previous focal necrosis, inflammatory reactions, or tissue repair were found in the region that had received cell injections. Inside the fibrotic tissue, trichrome and picrosirius collagen staining disclosed regions with decreased collagen density, in which a rich vascular tree was present. The anterolateral wall also showed larger central vessels that ramified into smaller ones, parallel to the cardiomyocyte bundles (Figure 2 B). In the anterolateral wall, the peripheral zone of fibrotic areas merged into the

cardiomyocyte layer and lacked well-defined limits, unlike the fibrotic areas observed in the posterior wall ([Figure 2C](#)). No fibrotic tissue was seen in the central area of the septal wall ([Figure 2D](#)).

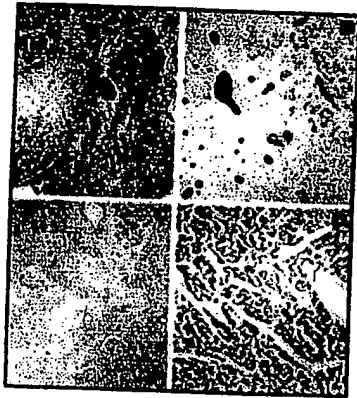


Figure 2. Gomori trichrome stain of anterolateral (A, B), posterior (C), and septal (D) walls. Increased vascular tree is present in B. Original magnification is x40 in A, B, and D; x100 in C.

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Inflammatory cells were rare in the perivascular region: There were occasional isolated small groups of lymphocytes and, very rarely, granulocytes. At the interface between fibrotic tissue and cardiomyocyte bundles, 2 gradients merged: the decreasing blood vessel diameter and the increasing cardiomyocyte size. Very small cardiomyocytes were seen isolated in the fibrotic matrix adjacent to capillaries in the anterolateral wall, together with a progressively increasing number of fibroblastoid cells that were isolated or interspersed in small groups among the cardiomyocyte bundles.

Immunocytochemistry Findings

Immunocytochemical labeling of factor VIII-associated antigen identified a thin endothelial layer of blood vessels in the posterior, septal ([Figure 3A](#)), and anterolateral ([Figure 3B](#)) ventricular walls. In the anterolateral wall, neither factor VIII nor CD34 was found in the fibroblastoid cell population inside the fibrotic matrix. In the posterior ventricular wall and septum, smooth muscle α -actin was readily identified in blood vessel wall cells. This protein was present both in pericytes and in the smooth muscle cells of the thin vessel wall layer ([Figure 3C](#)) in the anterolateral wall. The vascular tree of the anterolateral wall showed intense labeling in the blood vessel walls, which had a marked hypertrophy of smooth muscle cells ([Figure 3D](#)). The same staining pattern was present in isolated cells located in the perivascular position and in the adjacent region among cardiomyocytes and fibrotic matrix ([Figure 3E](#)). Vimentin was present in the endothelial layer of the anterolateral wall, in the perivascular cells, and in cells adjacent to or in close contact with the cardiomyocytes ([Figure 4A](#)). These cells frequently formed an extensive network that permeated the fibrotic matrix and the

interstitial space among cardiomyocytes (Figure 4B).

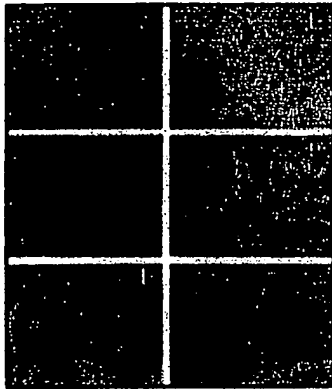


Figure 3. Immunocytochemical identification of factor VIII-associated antigen (A, B) and smooth muscle α -actin (C-E) in blood vessel walls of septal (A) and anterolateral (B-E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C-E). Ki67 reactivity was rarely present in perivascular cells of anterolateral wall (F). Original magnification x40 in A and B, x400 in C and D, and x1000 in E and F.

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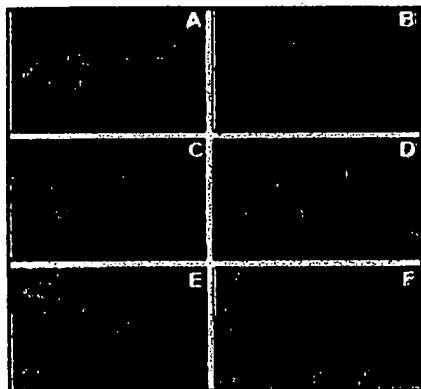


Figure 4. Anterolateral wall that received cell injection therapy. A and B, Immunostaining for vimentin depicted positive reaction in vascular wall and in fibroblastoid interstitial cells. C and D, Immunostaining for desmin showed small groups of intensely reactive cells between blood vessels and cardiomyocytes (C) and small cells inside cardiomyocyte bundles with typical striated cytoskeleton (D). E and F, Immunostaining for troponin showed positive reaction in all mural cells of medium-sized blood vessel. Original magnification is x1000 in A and F; x400 in B-E.

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Desmin was identified in the same cell population. Desmin labeling was less intense in the vascular wall cells and isolated perivascular cells and was more intense in the cells adjacent to cardiomyocytes. On sections perpendicular to the main cardiomyocyte axis, thin desmin-positive fibrils were observed mainly in the submembrane region; on longitudinal sections, a typical transverse banded pattern of desmin was observed (Figure 4C and 4D). Among cardiomyocytes, some

of the small cells had strong, peripheral desmin-stained areas (Figure 4D).

In vascular and perivascular cells in the posterior wall and septum, troponin labeling was negative. In the anterolateral wall, troponin labeling was negative in capillary walls but was positive in the adjacent pericapillary pericytes and in cells migrating into the pericapillary matrix (Figure 4E). In larger vessels, troponin-positive cells were observed in the outer cell layers and adventitia, occasionally forming a continuous troponin-positive cell layer around the vessel (Figure 4F). Isolated cells or small groups of troponin-positive cells were found in the area between the fibrotic tissue and cardiomyocytes and inside the adjacent cardiomyocyte bundles. The intensity of labeling increased in the proximity of cardiomyocytes, where some small fibroblastoid cells disclosed a bright cytoplasm homogeneously labeled for troponin (Figure 5A). Occasionally, such cells had an increased volume, with troponin labeling restricted to the periphery; the central area was filled by a troponin-negative cytoskeleton similar to the desmin-stained areas in small cardiomyocytes. In mature cardiomyocytes, troponin-specific antibody labeled the peripheral filamentous and sarcomeric cytoskeleton.

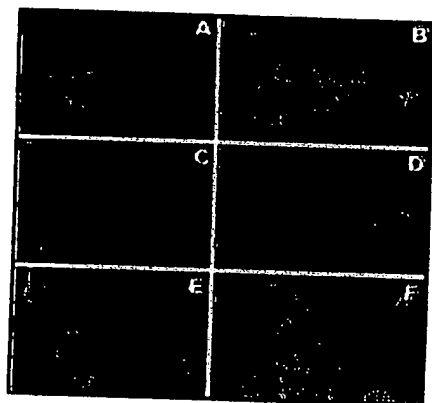


Figure 5. Anterolateral wall that received cell injection therapy. A, Immunostaining for troponin depicted small cardiomyocyte-like cells with intense reaction in peripheral cell area. B–F, Immunostaining for sarcomeric actinin depicted reactivity in mural cells of blood vessel (B–E) and isolated cells among cardiomyocytes with actinin organization similar to that of sarcomeres (E, F). Original magnification is x400 in A and F; x1000 in B–E.

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Labeling of sarcomeric actinin was similar to that of troponin. However, both pericapillary pericytes and mural blood vessel cells in the anterolateral region were negative for sarcomeric actinin in blood vessels that were deeply embedded in the fibrotic scar matrix and that remained distant from cardiomyocyte bundles. The same cells located in vessels adjacent to or embedded between the cardiomyocyte bundles were positive for sarcomeric actinin, as were the isolated cells or small groups of fibroblastoid cells (Figures 5B and 5C). Some of these cells had increased in size and, in their central region, disclosed sarcomeric actinin that was already organized in the typical banded pattern of sarcomeres (Figures 5D and 5E). In this central region, isolated cells barely larger than pericytes

could be observed; only a few sarcomeres were present, suggesting that those isolated cells had acquired some cardiomyocyte characteristics (Figure 5F).

The Ki67 antibody, which identifies cells actively engaged in replication, reacted only rarely with endothelial cells in the posterior wall. In the anterolateral region, the Ki67 antibody also reacted with pericapillary pericytes and with isolated fibroblastoid cells in the surrounding fibrotic matrix (Figure 3F). The overall cell reactivity with Ki67 antibody was relatively low.

► Discussion

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Accumulating evidence from both experimental animal studies⁴⁻⁶ and human trials⁷⁻⁹ indicates that ABMM cell therapy improves myocardial perfusion in patients with ischemic heart disease. At the same time, clinical stem cell therapy research is focusing more on safety than on efficacy. The present report describes the postmortem study of one patient who underwent transendocardial injection of ABMM cells. Accordingly, the major findings in this report pertain to the procedure's safety: No abnormal or disorganized tissue growth, no abnormal vascular growth, and no enhanced inflammatory reactions were observed. In addition, some intriguing histological and immunohistochemical findings were documented: (1) There was a higher capillary density in the cell-treated area than in nontreated areas of the heart. (2) A proliferation of smooth muscle α -actin-positive pericytes and mural cells was noted. (3) The aforementioned cells expressed specific cardiomyocyte proteins.

In the postnatal period, new blood vessels form through either vasculogenesis or angiogenesis, in which proliferation of endothelial cells is followed by remodeling of the extracellular matrix and proliferation of blood wall cells.¹³⁻¹⁵ Endothelial cells can result from bone marrow-derived progenitors (postnatal vasculogenesis) or from the migration and proliferation of endothelial cells from existing vessels (angiogenesis).¹⁶ Mural cells such as pericytes and smooth muscle cells can be derived from bone marrow mesenchymal cells (stroma), myofibroblasts, and/or fibroblasts.¹⁷ In the neoangiogenic process, pericytes are derived either from cells of adjacent tissues (mobilized by growth factors produced by endothelial cells) or from proliferation of adventitial and pericapillary pericytes and their distal gliding on the abluminal side of the growing blood vessel's basement membranes.¹³ The alternative origination of pericytes from mesenchymal stem cells has been proposed and preliminarily confirmed in experimental models.¹⁸ Pericytes may be essential to achieve a physiological angiogenic process with resultant durable blood vessels. In the present case, when compared with the noninjected regions, the cell-injected wall had marked hyperplasia of pericytes and mural cells. The observed hypertrophic pericytes displayed 2 characteristics: First,

although still located in the vascular wall, they expressed specific myocardial proteins and second, they were found in locations that suggested detachment, having migrated into the adjacent tissue and reached proximal cardiomyocytes that were either isolated or in small cell clumps. Closer to cardiomyocytes, the expression of myocardial proteins was enhanced, yielding brighter immunostaining throughout the whole cytoplasm. The significance of these findings remains to be established. However, within the posterior wall, none of the findings was seen, and small blood vessels could only rarely be found.

Notwithstanding the aforescribed data, the present report has limitations that severely restrict our ability to make conclusions about the role of ABMM cells in myocardial regeneration. The findings could have occurred by chance. It is impossible to exclude the influence of a natural recovery process as the cause for the difference in vascular density between cell-treated and nontreated areas. Comparisons of capillary density among different sections of wall were based on specimens from a single patient. Moreover, this is an isolated, uncontrolled case involving late events after injection of unlabeled cells; it precluded the use of any imaging technique that could have helped to colocalize and identify the presence of stem cell direct descendants within the vessel wall or myocardium. Therefore, the significant difference in vascular density between cell-treated and nontreated areas cannot be extrapolated to a larger population of similar patients. However, the increased vascular density within the cell-injected anterolateral wall accompanied that wall's improvement in perfusion as assessed by SPECT, whereas all other walls remained unchanged.

► Conclusion

At 11-month follow-up evaluation, stem cell therapy was not associated with any adverse histological findings. Morphological and immunohistochemical analysis of the area that underwent ABMM cell implantation suggested that that area had more capillaries than nontreated areas and that ABMM cell therapy was associated with hyperplasia of pericytes, mural cells, and adventitia. Some of these cells had acquired cytoskeletal elements and contractile proteins (desmin, troponin, and sarcomeric actinin).

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► Acknowledgments

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assistance with statistical questions.

► Footnotes

*Drs Dohmann and Perin are coprincipal investigators. †

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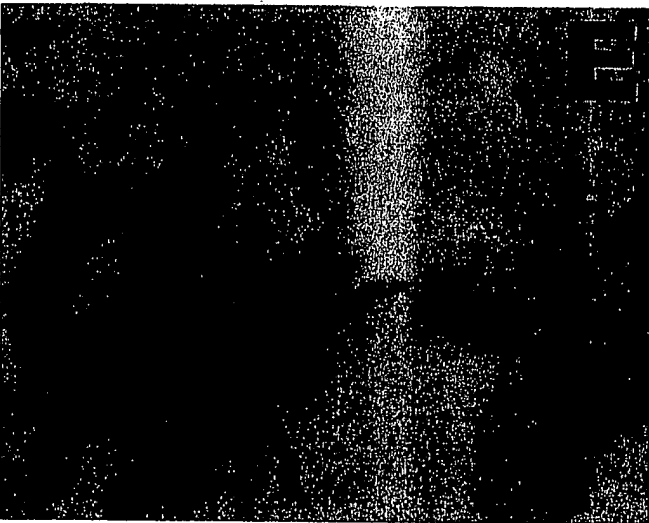
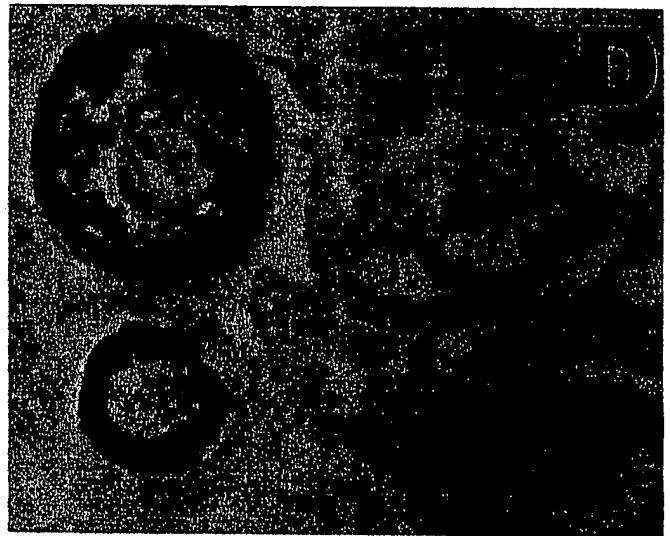
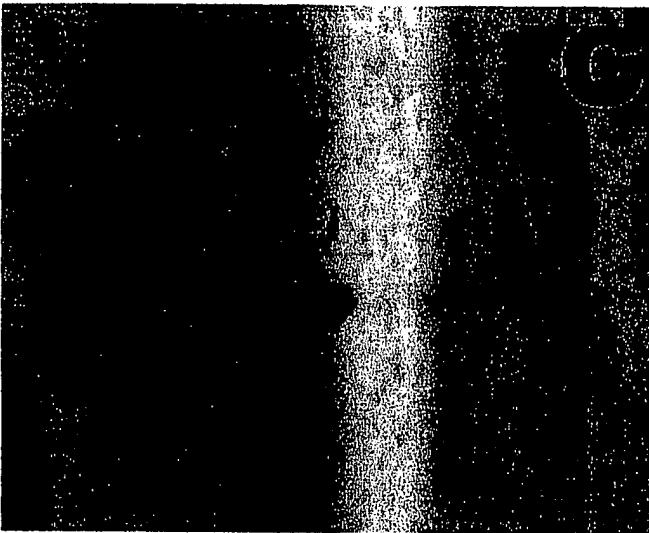
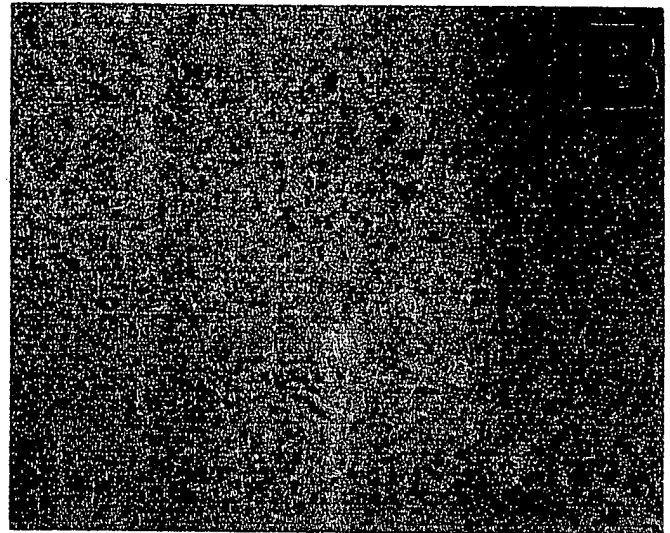
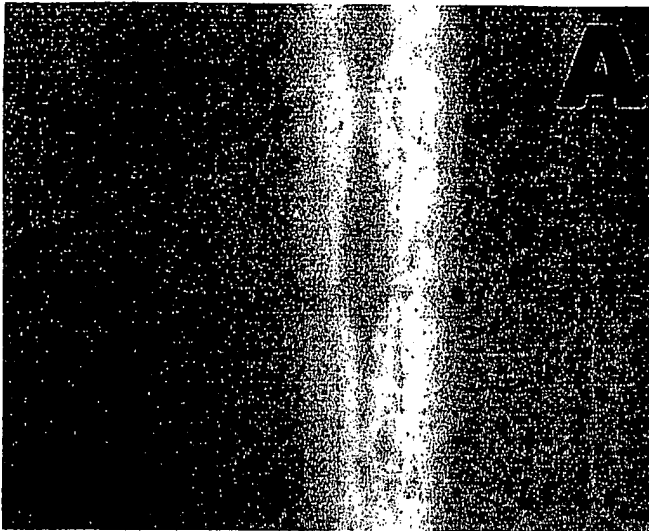
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Blood, April 15, 2007; 109(8): 3147 - 3151.

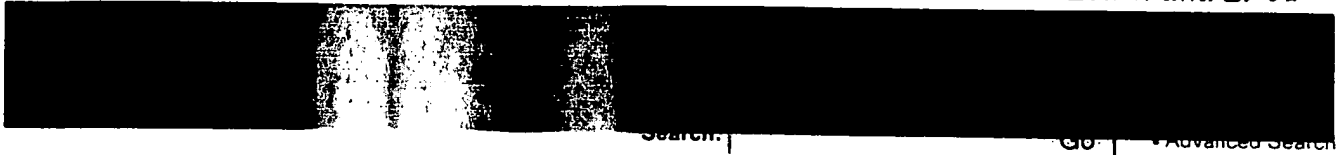
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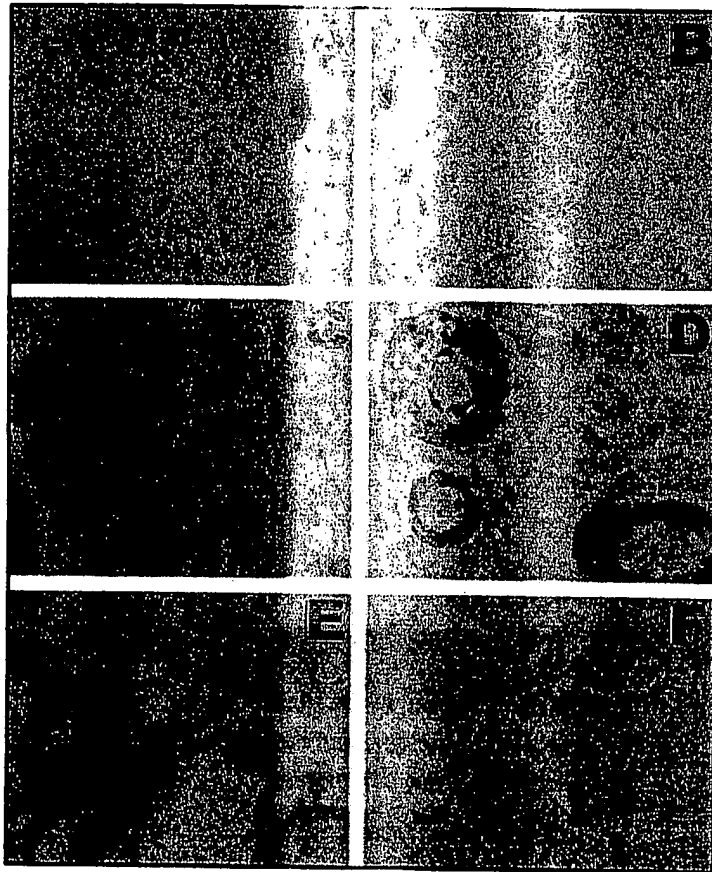


Figure 3. Immunocytochemical identification of factor VIII-associated antigen (A, B) and smooth muscle α -actin (C–E) in blood vessel walls of septal (A) and anterolateral (B–E) regions of studied heart, depicting increased vascular density (B) and hyperplasia of perivascular and mural cells (C–E).

EVIDENCE APPENDIX

ITEM NO. 8

**July 1, 2005 publication in
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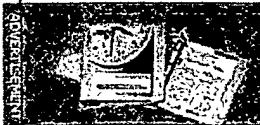


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Speaker: David Holmes, MD

Moderator: Reginald Low, MD

Panel members: George Dangas, MD, Wolfgang Ritter, MD, Peter Gonschior, MD,
Brian Firth (Cordis Corporation)

George Dangas: There have been many attempts and many failures in this field due perhaps to the inherent tendency of interventional cardiologists to move quickly and work from assumptions, applying what may not yet be well understood. Gene therapy and engineered viruses (associated viruses, attenuated viruses, etc.) are examples of this. When it became clear that we were unable to identify the most appropriate and effective agent for angiogenesis, we looked toward the newly fashionable stem cell-based therapies. Even researchers make 3 million agents, and 2 of them turn out to be effective, that would be fine. On the other hand, perhaps the stem cells will produce 2 or 3 agents that work for angiogenesis, but at the same time, 1 or 2 other agents produce negative effects — the result being that the positive effect hoped for is not achieved. Thus, the interventional cardiology field must achieve more "crisp" results based on more "crisp" basic science, with better-established findings, in order to better understand what the targets are and pursue them in a more methodological manner. Our methodology needs to be evidence-based, as opposed to the focusing on the practicalities of how to achieve our aims. We need to scale down the in vivo applications and return to the laboratory.

David Holmes: There are a number of small, randomized trials currently under way, primarily in Europe. Perhaps some of our European colleagues here could discuss these trials. We are already in the middle of human trials before obtaining adequate scientific data about which specific cells to use, how many cells, when to deliver them, and how to deliver them. Is that a good thing? What if they fail? does that mean the approach is wrong? Or does it mean that we were doing it incorrectly?

Peter Gonschior: The good thing is that very robust cells are used based on solid, basic scientific data. That led to the application of a large variety of cells, which led to what appeared to be good data. The patient data, such as ejection fraction, however, are not terribly impressive. Ejection fraction improvement is not very significant, especially when you factor in the amount of energy wasted to achieve any clinical impact in the patients. More basic, relevant data are required to guide us toward the best approach.

Wolfgang Ritter: We have never used drug-eluting stents, so we wait to see what the cardiologist does.

David Holmes: From the industry standpoint, it would be nice to have a patentable product so that the product's unique design bearing the corporate name could be marketed. Given that God invented the progenitor cells and has a pretty strong patent on them, how do we "patent" a cell? For example, a bone marrow cell injected in the coronary artery — how do you design a device to do that? How do you make a living at that, since most any device could do that task?

Brian Firth: Let me come at this from a different angle. For some time now, Cordis has looked at what it already had as facilitating technology. We have been on the delivery side of the business, specifically with our Noga systems, the NogaStar®, the MyoStar™ injectable catheter, and so on. Thus, the mapping, definition and ability to deliver something in a very site-specific manner constitute the piece of the business Cordis has focused on. Having said that, in order to obtain 510-K FDA device approval, we must prove that it actually does something. Thus, Cordis is currently working on the area of autologous bone marrow with stem cells. Our interest is not in trying to figure out how to patent stem cells, which can't be done, but rather in the delivery of these cells, because we think that a more local delivery system would be better than a more general one. Cordis seeks to design a system, thus, that would deliver the cells that have been identified for their contractile properties to a site that has been defined as compromised.

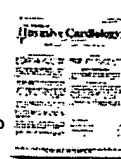
Richard Heuser: These are expensive studies to conduct, thus, if the product is not patentable, it will not attract industry funding. In the case of the Bioheart study, how will this trial be conducted? Will sham cells be given, or no cells, or a small number of them? Also, we want to target the patient population that is not eligible for heart transplants. We have been talking about bone marrow cells as well. My understanding is that there is a very good possibility that these cells can be delivered intravenously with the same results. So how do we design, say, a skeletal muscle cell study that would actually end up garnering FDA approval for the therapy? And what about bone marrow — is it really necessary to go down the coronary arteries and go selectively into the myocardium?

David Holmes: Those are two important questions. Bioheart is a skeletal muscle myoblast product. The company considered this product a drug when it applied to the FDA for approval. In drug trials, the FDA requires data on ineffective dosage in addition to a toxic dose, and a couple of doses that do work. Thus, the first dose in the Bioheart project that was approved by the FDA was absolutely ineffective — it might as well have been placed under the patient's pillow. The data that came out of Europe on Bioheart involved a much higher dose, albeit with a small number of patients.

In terms of the second point as it relates to where and how to administer the cells, some information has shown that when these different sorts of cells are delivered intravenously, they go to the lungs and have a "tremendous time," and they don't reach the myocardium. So while it makes perfect sense to use the intravenous approach, these cells are filtered out in the lungs and remain there. If those cells are active and produce cytokines, perhaps that's all we would want to use them for. Maybe these cells aren't the magic solution, and maybe we don't have a clue about this. Perhaps we can use these cells for the cytokines they produce systemically and they will cause other bone marrow cells to hone in on the site of injury. But at the present, we just don't know enough about this process.

Patrick Whitlow: I just want to give you an update on Bioheart because of their underlying disease process, these patients are very prone to arrhythmia and sudden death. And theoretically, if you are adding islands of tissue in the left ventricle that is already damaged, these islands of tissue are not enervated in the same way as the surrounding tissue and the conduction properties aren't the same. You would theorize that this could set up re-entry circuits. Thus, ventricular arrhythmia presents an enormous problem in terms of conducting studies because many of these patients are going to die from their underlying disease. To detect if cell injection causes worsened arrhythmias will be very difficult, but a potentially serious problem. Therefore, the first v.s. clinical trial involves patients who already have defibrillators, and the number of patients will be small because of the need for defibrillators. The study should answer the question of whether this is arrhythmogenic — which Patrick Serruys believes is the case. Other researchers in France don't believe that injecting cells is arrhythmogenic. Who knows? It will take a long time and a lot of patients to arrive at the answer.

If a start-up company tries to make this therapy work, it will be very difficult for industry to actually fund the research from start to finish. We know from the animal studies that efficacy increases with higher doses of cell therapy, but we have yet to find what a potentially toxic dose is for the size of the island of

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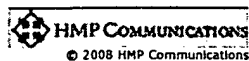
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EVIDENCE APPENDIX

ITEM NO. 9

**Murry et al. 1996 publication in J. Clin. Invest. entitled,
“Skeletal Myoblast Transplantation for Repair of Myocardial
Necrosis” cited by the Examiner in the November 28, 2003
Office Action**

Skeletal Myoblast Transplantation for Repair of Myocardial Necrosis

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Abstract

Myocardial infarcts heal by scarring because myocardium cannot regenerate. To determine if skeletal myoblasts could establish new contractile tissue, hearts of adult inbred rats were injured by freeze-thaw, and $3\text{--}4.5 \times 10^6$ neonatal skeletal muscle cells were transplanted immediately thereafter. At 1 d the graft cells were proliferating and did not express myosin heavy chain (MHC). By 3 d, multinucleated myotubes were present which expressed both embryonic and fast fiber MHCs. At 2 wk, electron microscopy demonstrated possible satellite stem cells. By 7 wk the grafts began expressing β -MHC, a hallmark of the slow fiber phenotype; co-expression of embryonic, fast, and β -MHC continued through 3 mo. Transplanting myoblasts 1 wk after injury yielded comparable results, except that grafts expressed β -MHC sooner (by 2 wk). Grafts never expressed cardiac-specific MHC- α . Wounds containing 2-wk-old myoblast grafts contracted when stimulated ex vivo, and high frequency stimulation induced tetanus. Furthermore, the grafts could perform a cardiac-like duty cycle, alternating tetanus and relaxation, for at least 6 min. Thus, skeletal myoblasts can establish new muscle tissue when grafted into injured hearts, and this muscle can contract when stimulated electrically. Because the grafts convert to fatigue-resistant, slow twitch fibers, this new muscle may be suited to a cardiac work load. (*J. Clin. Invest.* 1996; 98:2512–2523.) Key words: myocardial infarction • skeletal myoblast • myosin heavy chain • contractile function • cell transplantation

Introduction

Experimental and clinical therapies for myocardial infarction have focused traditionally on limiting infarct size. Unfortunately, the goal of limiting myocardial injury has been difficult to achieve clinically, because ischemic myocardium dies quite rapidly (1) and most patients wait more than 3 h after coronary occlusion before seeking medical attention. As an alternative approach, we are exploring strategies to induce the injured heart to heal with muscle replacement rather than forming scar tissue.

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One strategy for muscle regeneration is to transplant either skeletal or cardiac myocytes into the injured heart. Studies from Field's group showed that cardiac myocytes can be transplanted into normal hearts, where they couple with host cardiocytes via intercalated discs (2, 3). However, a major drawback to using cardiocytes is their inability to proliferate in culture. At present it seems unlikely that enough primary cardiocytes could be obtained from the patient or histocompatible donor to repair a myocardial infarct in humans. On the other hand, skeletal muscle satellite cells (muscle stem cells) proliferate well in culture. Satellite cells could be obtained from muscles of infarct patients and rapidly expanded in culture, or stocks of potentially therapeutic myoblasts could be obtained from embryos and frozen for subsequent use (4, 5). Furthermore, physiological studies have shown that when properly conditioned, skeletal muscle can adapt to perform a cardiac-type work load (6). Recent studies have demonstrated the feasibility of grafting skeletal myoblast lines into normal hearts (7) and autologous satellite cells into injured hearts (8, 9). However, to generate significant amounts of functional new muscle the transplanted cells ideally should proliferate and then differentiate into mature myofibers capable of sustaining a cardiac work load. This study was performed to determine the proliferation and differentiation patterns of skeletal myoblasts after engraftment into injured rat hearts and to determine whether this new muscle could support contractile activity.

Methods

Skeletal myoblast isolation and culture. These studies were approved by the University of Washington Animal Care Committee and were conducted in accordance with federal guidelines. Skeletal myoblasts were obtained from the limbs of 1–3-d-old Fischer rats. This inbred strain was used to avoid immune barriers to transplantation. After time of killing, the carcasses were skinned and the limbs were placed into cold tissue culture media. Under a dissecting microscope, the muscles were stripped of surrounding adipose tissue and fascia and bluntly dissected from their tendons. The muscles were minced with iridectomy scissors until a fine slurry was formed. The slurry was then digested in 0.05% trypsin/EDTA (GIBCO-BRL, Gaithersburg, MD) in Ham's saline A at 37°C, with intermittent mechanical agitation to assist dispersal. After 30–45 min the cell suspension was filtered through sterile gauze to remove undispersed tissue fragments and rod shaped mature myofibers. Cells were plated at $\sim 5 \times 10^6$ cells/dish in 100-mm gelatinized plates in 10 ml Ham's F10C media, containing 15% horse serum and 50 μ g/ml gentamicin sulfate (Gemini Bioproducts, Inc., Calabasas, CA). Recombinant human basic fibroblast growth factor was added twice daily to a final concentration of 6 ng/ml, and the complete medium was replaced once per day. Approximately 10% of the cells attached and grew with a doubling time of ~ 18 h. The cultures contained a mix of small, oval myoblasts and elongated, spindle-shaped cells consistent with fibroblasts. Subconfluent cultures were passaged every 2–3 d (1:5 split) to minimize the occurrence of myogenic differentiation at higher density. On the day before transplantation, the cultures were tagged for subsequent identification in vivo. In some experiments cells were tagged with fluorescent micro-

spheres (1:500 dilution of stock 200 nm yellow-green fluorescent microspheres; Molecular Probes, Eugene, OR). The latex microspheres were endocytosed (typically > 20 spheres/cell) and served as cytoplasmic markers (10). In other experiments, cells were incubated overnight with [³H]thymidine (1 μ Ci/ml) to mark their nuclei after autoradiography. Cultures were trypsinized immediately before transplantation and suspended at a concentration of $\sim 3 \times 10^6$ /ml. Small aliquots of the remaining cell suspension were replated at $\sim 2 \times 10^4$ cells/cm² into gelatinized, multichamber plastic slides, and fixed in methanol after various culture intervals for immunostaining.

Rat cardiac injury models. Inbred male Fischer rats (Simonsen Labs, Gilroy, CA) weighing 350–400 grams were anesthetized with intraperitoneal ketamine-xylazine (68 and 4.4 mg/kg, respectively), intubated, and mechanically ventilated with room air. The heart was exposed aseptically via a left thoracotomy, and a 1-cm-diameter aluminum rod, precooled with liquid nitrogen, was placed in direct contact with the anterior left ventricle for 15 s. Freeze-thaw reproducibly caused a disc-shaped region of coagulation necrosis, ~ 1 cm in diameter, extending ~ 2 mm into the myocardium. It should be noted that while infarcts typically have irregular borders with viable peninsulas of subepicardial myocardium along penetrating vessels, freeze-thaw lesions consist of confluent necrosis in the subepicardium with viable myocardium in the subendocardium. Because they are not transmural, freeze-thaw lesions do not cause cardiac aneurysm formation. Despite these differences, the cellular patterns of coagulation necrosis, inflammation and phagocytosis, granulation tissue formation, and scarring after freeze-thaw injury are indistinguishable from myocardial infarction (11–13), making it a suitable model to study myocardial repair.

In the initial studies, $\sim 3 \times 10^6$ myoblasts in 100 μ l tissue culture media were injected superficially into the center of the injured region immediately after injury, using a 27-gauge needle. Then, the chest was closed and the rats were allowed to recover for timed intervals from 1 d to 3 mo ($n = 4$ /time point). To mimic a clinical situation more closely, a second protocol was used in which the freeze-thaw lesion was allowed to heal for 1 wk before transplanting myoblasts. By 1 wk, most of the necrotic myocardium had been replaced by granulation tissue, but scar formation had not yet begun. The rats ($n = 2$ /time point; no 3 d or 3 mo time points) were reanesthetized and a thoracotomy was repeated. The heart was exposed and a 100- μ l suspension containing $\sim 3 \times 10^6$ myoblasts was injected into the wound as described above. The chest was closed and the animals were allowed to recover for intervals from 1 d to 7 wk.

To detect DNA synthesis in the grafts the rats were treated with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Boehringer-Mannheim, Indianapolis, IN). 1 d before time of killing, the rats were lightly anesthetized, and a 50-mg tablet of BrdU was implanted subcutaneously for measurement of cell replication. Preliminary studies showed that a subcutaneous 50-mg BrdU tablet gave comparable replication rates to a 24-h continuous infusion with an osmotic mini-pump (not shown). For rats killed 1 d after transplantation, a single 10-mg pulse of BrdU was given intraperitoneally 1 h before time of killing. This avoided incorporation of BrdU into the cells which were cycling at the time of transplantation.

Rats were killed with a pentobarbital overdose and their hearts were excised. In the immediate transplantation groups, the aorta was cannulated and the hearts were perfused fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid), transversely sectioned, and embedded in paraffin by routine methods. In groups transplanted 1 wk after injury, the hearts were transversely sectioned, embedded in OCT (Miles Inc., Kankakee, IL), and frozen in a dry ice-ethanol bath for frozen section analysis. In both protocols, sections of gut were obtained as controls for measurement of cell replication with BrdU.

Abbreviations used in this paper: BrdU, 5-bromo-2'-deoxyuridine; MHC, myosin heavy chain.

Measurement of contractile function in isolated wound strips. Rat hearts were given 4.5×10^6 myoblasts ($n = 8$) in 100 μ l or a sham injection of saline ($n = 3$) immediately after injury. 2 wk after engrafting, the hearts were excised and transversely sectioned. Under a dissecting microscope, most of the subendocardial myocardium was trimmed away from the injured region, and isolated wound strips ($\sim 1.5 \times 1.5 \times 8$ mm) were prepared. One or two strips were studied from each myoblast-engrafted heart, and two or three strips were studied from each sham-injected heart. The strips were ligated at both ends with silk suture and then placed in a bath of physiological saline with the following composition (mmol/liter): 116 NaCl, 4.6 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 26 Mops (pH 7.4), 11 glucose, and 10 mg/liter gentamicin. The buffer was equilibrated with 95% O₂/5% CO₂ and maintained at 20°C via a thermostatically controlled water jacket. Wound strips were mounted between an isometric force transducer (model 60-2995; Harvard Apparatus, Inc., South Natick, MA) and a fixed glass hook. Resting tension was set initially at 0.5 g. Strips were stimulated with 1-ms bipolar pulses delivered via platinum wire electrodes using a Grass model S48 stimulator (Astro-Med, Inc., West Warwick, RI). Voltage was increased in 10-V increments until contractile activity was observed. Force traces were displayed on a digital storage oscilloscope (model 3091; Nicolet Instrument Corp., Madison, WI) and recorded using a General Scanning model RS4-5P strip chart recorder. After determining the force-voltage relationship, the optimal length for force production was determined for each wound strip using test contractions at 2-min intervals, a time sufficient for metabolic recovery in mammalian fast twitch muscles (14). Force-frequency analysis was performed by increasing the stimulation frequency in 1-Hz increments; tetanus was defined as the point where the oscillations of contractile force at the plateau were < 3% of the net force generated (14). Finally, to test fatigability the grafts were subjected to a simulated cardiac-like duty cycle, consisting of 0.33 s of tetanus followed by 0.67 s of relaxation (1:2 cycle), continuing for 6 min. After completion of functional studies the strip's cross-sectional area was determined, and the tissue then was processed for histology or electron microscopy.

Immunocytochemistry. Antibodies used for immunostaining are given in Table I. 6- μ m frozen sections were cut on a cryostat, briefly air dried, and stored at -70°C until use. 5- μ m paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Cultured cells were fixed and stored in cold PBS until use. For all samples, endogenous peroxidase activity was quenched by incubating with 0.3% H₂O₂ in methanol for 30 min. Immunostaining was carried out at room temperature. Sections were blocked with 1.5% normal horse serum in PBS for 1 h. The sections were then incubated with the primary antibody in 1.5% horse serum for 1 h, followed by incubation with the secondary antibody (rat adsorbed horse anti-mouse, 1:400 dilution; Vector Labs, Inc., Burlingame, CA) for 1 h. Antigens were localized with an avidin-biotin-peroxidase complex (ABC Elite kit; Vector Labs). For staining with a single antibody, diaminobenzidine (Sigma Immunochemicals, St. Louis, MO) was used as a chromagenic substrate. For double immunolabeling with antibodies to myosin and BrdU, sections were first exposed to 1.5 N HCl for 15 min at 37°C to denature the DNA, followed by a rinse in 0.1 mol/liter borax to stabilize the denatured strands. Sections were then stained routinely for myosin heavy chain (MHC) using diaminobenzidine. After a second quenching in 0.3% H₂O₂, sections were blocked with 1.5% normal horse serum, and then incubated with a mouse monoclonal antibody to BrdU for 1 h. After incubation with the secondary antibody (horse anti-mouse), BrdU was localized with an avidin-biotin-peroxidase complex, using True Blue (KPL, Gaithersburg, MD) as substrate. Cross-reactivity between the first primary antibody and the second secondary antibody did not occur, as long as the True Blue substrate was incubated for a short duration (< 1 min). Sections were counterstained either with methyl green, nuclear fast red, or hematoxylin.

Electron microscopy. After measurement of contractile function, one of the tissue strips was immersed in half strength Karnovsky's fix-

Table 1. Antibodies Used for Immunocytochemistry

Antibody	Antigen recognized	Dilution	Source	Reference
MF-20	Sarcomeric MHCs	Hyb. Sup., 1:100	American Type Culture Collection, Rockville, MD	39
MY-32	Skeletal MHC-fast (types IIA and IIB)	Mouse ascites, 1:2000	Sigma Immunochemicals	40
BA-G5	Cardiac MHC- α	Hyb. Sup., 1:5	American Type Culture Collection	41
F1.652	Embryonic MHC	Hyb. Sup., 1:100	Developmental Studies Hybridoma Bank*	42
A4.951	β -MHC	Hyb. Sup., 1:50	American Type Culture Collection	43
Anti-BrdU	BrdU	IgG, 1:50000	Eurodiagnostics, Apeldoorn, The Netherlands	44

IgG, purified IgG monoclonal antibody; Hyb. Sup., hybridoma supernatant. * The monoclonal antibody F1.652, developed in the laboratory of Dr. Helen Blau, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract NOI-HD-2-3144 from the National Institute of Child Health and Human Development.

active and dissected into small cubes < 1 mm in greatest dimension. The tissue was fixed overnight in half strength Karnovsky's fixative, postfixed for 1 h in 1% osmium tetroxide at room temperature, dehydrated through a graded alcohol series followed by propylene oxide, and embedded in Medcast resin (Ted Pella, Inc., Redding, CA). Semithin sections were stained with toluidine blue and examined by light microscopy. Thin sections were cut from selected blocks, stained with lead citrate and uranyl acetate, and examined in a Jeol JEM 1200EXII transmission electron microscope. Representative areas were photographed.

Results

Characteristics of myoblast cultures. The muscle cultures contained a mixed cell population. At least 22% of the cells were skeletal muscle, as indicated by their staining for sarcomeric myosin after switching to a differentiation medium containing 1.5% serum and no FGF for 3 d. This procedure underestimates the true percentage of skeletal muscle cells by several-fold, since the nonmyogenic cells continue to divide after the medium switch while the myoblasts complete their present cell cycle and then terminally differentiate. Approximately 1% of the cells stained with antibodies to smooth muscle α -actin, which can mark either smooth muscle cells or fibroblasts. Virtually none of the cells stained with an antibody for the endothelial marker von Willebrand factor. The remaining cells were presumably fibroblasts.

Histology and differentiation patterns of myoblast grafts. Cultured skeletal myoblasts were transplanted into cardiac freeze-thaw lesions either immediately after injury, or, to mimic a clinical situation more closely, cells were transplanted 1 wk after injury. The two protocols yielded similar results and will be described together; minor differences are noted below. On the first day after transplantation the myoblasts were mononuclear cells (Fig. 1 A). The grafted cells could be distinguished clearly from inflammatory cells within the necrotic tissue by their larger size and characteristic oval shape. (Fibroblast ingrowth from the surrounding tissue had not yet begun at this time.) The identity of the grafted cells was confirmed by their cytoplasmic fluorescent microspheres and radioactive nuclei (not shown). Mitotic figures were common. The grafted cells did not stain with antibodies to skeletal or cardiac MHCs (Fig. 1 B). Thus, muscle differentiation had not yet occurred.

By 3 d after transplantation, many of the grafted cells had fused to form multinucleated myotubes (Fig. 1 C). Myotubes were partially aligned along the short (transverse) axis of the

heart. The myotubes stained with antibodies to sarcomeric MHC, embryonic MHC (Fig. 1 D), and to MHC-fast (not shown). Occasional cross-striations were noted, but these were not frequent at this time (Fig. 1 D). The myotubes did not express cardiac MHC- α . By 1 wk the grafts were easily recognizable as skeletal myofibers and many cells contained cross-striations. As before, the new myofibers stained with antibodies to sarcomeric MHC, embryonic MHC, and MHC-fast, but did not express cardiac MHC- α (not shown). By 2 wk after transplantation the grafts had the appearance of maturing skeletal myofibers (Fig. 1 E). Sarcomeres were well formed, and many cells had peripheral nuclei. The myofibers stained intensely with antibodies to sarcomeric myosin, embryonic MHC (Fig. 1 F), and skeletal MHC-fast (Fig. 1 G). No staining with cardiac MHC- α antibodies was observed at 2 wk.

At 7 wk after transplantation the grafts were islands of mature skeletal muscle within young scar tissue (Fig. 1, H-J). There was a moderate increase in cell diameter compared with 2 wk. None of the muscle grafts were infiltrated or splayed apart by scar tissue, nor was there evidence of fiber atrophy. Vascular density appeared normal for muscle tissue (Fig. 1 J). All of the 7-wk grafts stained strongly with antibodies to sarcomeric myosin and embryonic MHC (Fig. 1 H). The grafts injected immediately after injury stained intensely with antiskeletal MHC-fast, comparable with Fig. 1 F. In contrast, the grafts injected 1 wk after injury stained poorly with antiskeletal MHC-fast (see below). No staining with antibodies to cardiac MHC- α was observed in the grafts, while the adjacent myocardium stained intensely (Fig. 1 I).

At 3 mo after transplantation the grafts again had the appearance of mature skeletal muscle (Fig. 1 K). Most myofibers had peripheral nuclei, and vascular density appeared normal. Fiber diameter was generally larger than in the 7-wk group, indicating that the cells had hypertrophied between 7 wk and 3 mo (compare Fig. 1, J and K). In one heart, however, part of the graft was infiltrated by scar tissue which encircled individual myofibers and was associated with fiber atrophy (Fig. 1 L). The grafts continued to express embryonic MHC and MHC-fast (not shown). Once again, no staining with antibodies to cardiac MHC- α was observed (comparable with Fig. 1 I). At all time points the myofibers were predominantly aligned parallel with the short (transverse) axis of the heart and therefore appeared in longitudinal section. However, some fascicles of muscle appeared obliquely or cross-sectioned in this plane.

In summary, the grafts began to differentiate into myo-

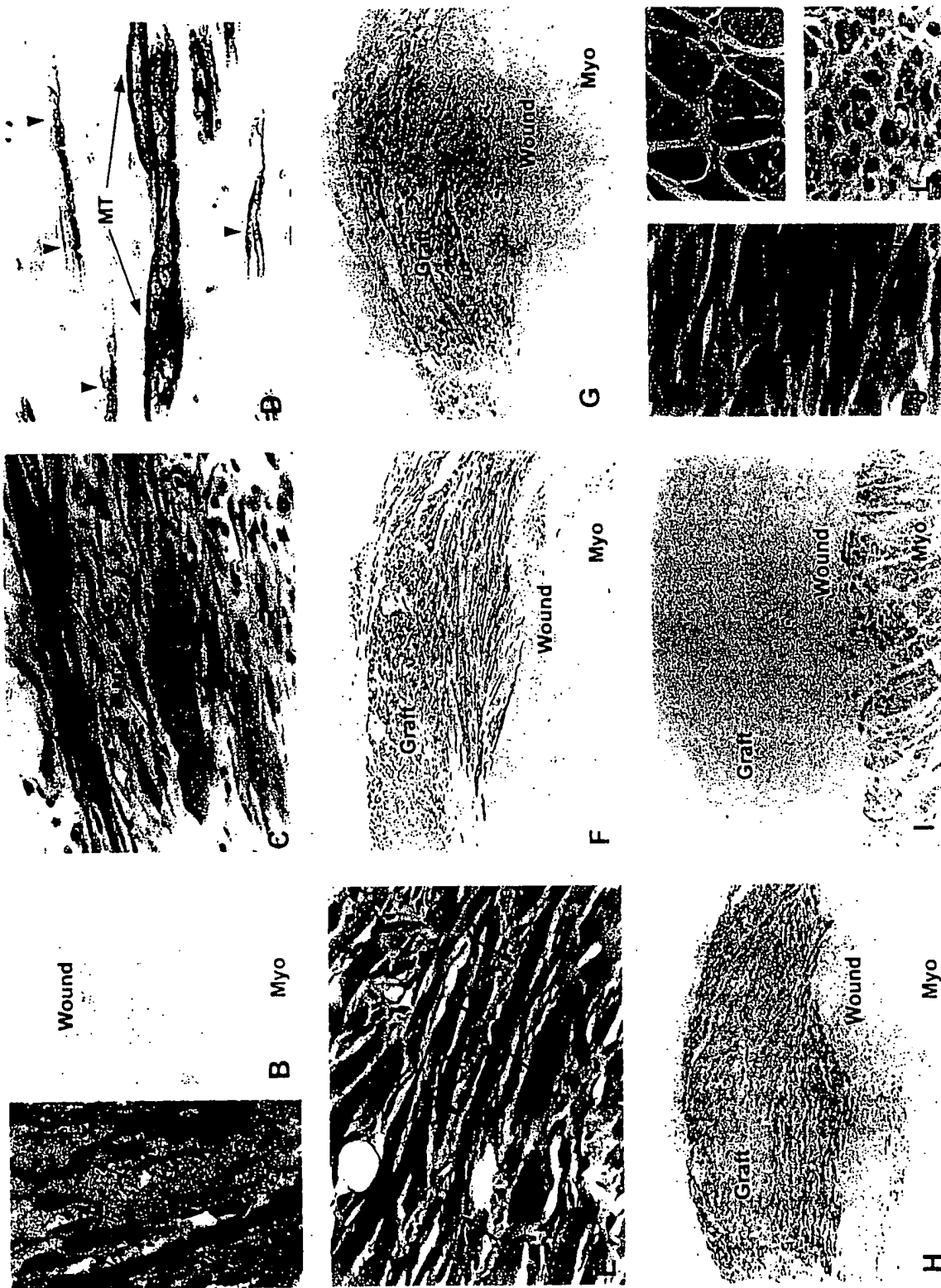


Figure 1. Morphology and MHC expression in skeletal myoblast grafts. Rat hearts were injured by freeze-thaw and syngeneic skeletal muscle cells were grafted into the lesions. All panels in this figure are from hearts which were grafted immediately after injury. (A) 1-d graft. The grafted cells are identifiable as relatively large, oval shaped cells (arrows) within the necrotic myocardium. One graft cell is in mitosis (arrowhead). Numerous smaller inflammatory cells are present within the lesion. Fibroblast ingrowth from surrounding viable tissue had not yet begun at this

tubes between 1 and 3 d and acquired the appearance of maturing myofibers with well formed sarcomeres by 2 wk. The grafts expressed both embryonic MHC and MHC-fast at all times between 3 d and 3 mo. There was no expression of cardiac MHC- α at any time.

Electron microscopy. Electron microscopy was performed on one heart, 2 wk after myoblast grafting. Most of the grafted cells had well formed, slightly contracted sarcomeres which were aligned in registry (Fig. 2 A). Mitochondria were abundant. Multinucleation was evident in many cells, as were well formed T-tubules. However, in other cells a spectrum of morphological stages was present, consistent with developing skeletal muscle (Fig. 2 B). Cells at the earliest stage were small, had scanty myofibril content, and contained focal aggregations of electron-dense material suggestive of developing Z-discs. In these cells there were abundant ribosomes and glycogen, a prominent Golgi apparatus, and dilated segments of sarcoplasmic reticulum. Intermediate cells were larger and had increasing amounts of myofibrils with a corresponding decrease in ribosomes and glycogen. Some cells had well formed sarcomeres, but these were out of registry compared with the most mature cells. No intercalated discs were identified between cells in the graft region. Adjacent myofibers often had intimately apposed, interdigitating cell membranes. Occasional cells were identified with electron-dense membrane structures suggestive of intermediate adherens junctions and gap junctions (Fig. 2, C and D). Some mature myofibers were closely associated with mesenchymal cells, located within the basal lamina compartment of the myofiber. Their location within the basal lamina of the myofiber suggests that they might be new satellite stem cells (Fig. 2, E and F). Some of these mesenchymal cells had abundant rough endoplasmic reticulum, similar to fibroblasts. Cells with this morphology have also been described in regenerating skeletal muscle by Trupin et al. (15). Their location within the basal lamina of the myofiber and the

absence of collagen in this space make it unlikely that these cells are actually fibroblasts.

Myoblast grafts convert from fast to slow twitch fibers. The poor staining for MHC-fast in the 7-wk group with delayed transplantation seemed at variance with the morphology of the grafts, which showed relatively hypertrophic cells with well formed sarcomeres. We hypothesized that the grafts had undergone fiber type conversion to slow twitch muscles, which no longer expressed high levels of MHC-fast. Slow twitch fibers have physiological similarities to cardiac muscle, including a high capacity for oxidative phosphorylation and fatigue resistance. Furthermore, slow fibers use β -MHC as a major contractile protein, which is also the predominant myosin in developing rat hearts. In contrast, fast twitch fibers use glycolysis for ATP production, have a low aerobic capacity and fatigue rapidly, and do not express β -MHC (16). Therefore, we compared β -MHC expression with skeletal MHC-fast, to determine fiber types in the maturing grafts.

At 1 wk the grafts stained intensely for MHC-fast (Fig. 3 A) but did not stain with an antibody to β -MHC (Fig. 3 B). At 2 wk the grafts continued to express MHC-fast. In the group transplanted immediately after injury no expression of β -MHC was noted at 2 wk, yet in grafts transplanted 1 wk after injury some cells expressed β -MHC (not shown). At 7 wk after transplantation the two groups differed in expression of MHC-fast, with strong staining in the immediate transplant group (see Fig. 1 G) and weak staining in the group where transplantation was delayed for 1 wk after injury (Fig. 3 C). However, both the immediate and delayed transplantation groups exhibited extensive staining for β -MHC at 7 wk after transplantation (Fig. 3 D). At 3 mo there was continued expression of β -MHC and MHC-fast in the immediate transplantation group; we did not study the delayed transplantation protocol at 3 mo. Thus, myoblast grafts appeared to be undergoing conversion from fast twitch to slow twitch fibers. Conversion appeared to take place

time. Hematoxylin and eosin stain. $\times 800$. (B) Low magnification of 1-d graft stained for embryonic MHC. The freeze-thaw lesion (Wound) occupies approximately the upper 75% of the field, while residual subendocardial myocardium (Myo) is present in the lower 25%. None of the grafted cells express embryonic MHC, indicating no differentiation had taken place yet. Methyl green counterstain. $\times 80$. (C) 3-d graft. Multiple multinucleated myotubes (MT) are present. Note that myotubes are already aligned in parallel. The surrounding tissue contains numerous fibroblasts (some of which may be of graft origin), macrophages, and capillaries, characteristic of granulation tissue. Two mitotic figures are present at the lower right (arrowheads). Hematoxylin and eosin stain. $\times 800$. (D) 3-d graft stained for embryonic MHC. The multinucleated myotubes (MT) express embryonic MHC, indicated by brown staining. Note faint cross-striations present at the periphery of some myotubes (arrowheads). Comparable staining was seen using antibodies to MHC-fast (not shown). Methyl green counterstain. $\times 800$. (E) 2-wk graft. Multinucleated myofibers are present and many have peripherally placed nuclei (arrows); most of these nuclei appear to be within the sarcolemma, although some may be immediately external. Cross-striations were readily seen under the microscope but appear faint in the photograph. Hematoxylin and eosin staining. $\times 800$. (F) 2-wk graft stained for embryonic MHC. The myofibers of the graft stain vigorously for embryonic MHC, while the underlying granulation tissue (Wound) and subendocardial myocardium (Myo) do not stain. Methyl green counterstain. $\times 80$. (G) 2-wk graft stained for fast fiber isoforms of MHC. There is intense staining of the engrafted myofibers (Graft), indicating that they exhibit a fast twitch phenotype. Note that the residual myocardium (Myo) beneath the graft does not stain, nor does the granulation tissue of the injured region (Wound). $\times 80$. (H) 7-wk graft stained for embryonic MHC. The graft continues to stain vigorously for embryonic MHC. There is no staining in the underlying young scar tissue (Wound) or the residual subendocardial myocardium (Myo). Methyl green counterstain. $\times 80$. (I) 7-wk graft stained for cardiac MHC- α . The skeletal myofibers of the graft do not express MHC- α , nor does the underlying scar tissue (Wound). This indicates that the grafted skeletal muscle does not show cardiac differentiation. The subendocardial myocardium (Myo) stains vigorously for MHC- α . Methyl green counterstain. $\times 80$. (J) 7-wk graft. Mature myofibers are present. Most myofibers have peripheral nuclei. Cross-striations were readily apparent under the microscope, but again are faint in the photograph. Multiple capillaries are present within the muscle tissue (arrows). Hematoxylin and eosin stain. $\times 800$. (K) 3-mo graft. The myofibers (obliquely and cross-sectioned) have peripheral nuclei and are closely apposed with little intervening extracellular matrix. The myofibers are hypertrophic compared with the 7-wk grafts (compare fiber diameter with J). Most 3-mo grafts had this appearance. Hematoxylin and eosin stain. $\times 800$. (L) 3-mo graft. The myofibers (cross-sectioned) in this region are encased by dense scar tissue and are atrophic. Note the markedly diminished cell diameters compared with K. Such entrapment of myofibers by scar was seen in one region of one heart. Hematoxylin and eosin stain. $\times 800$.

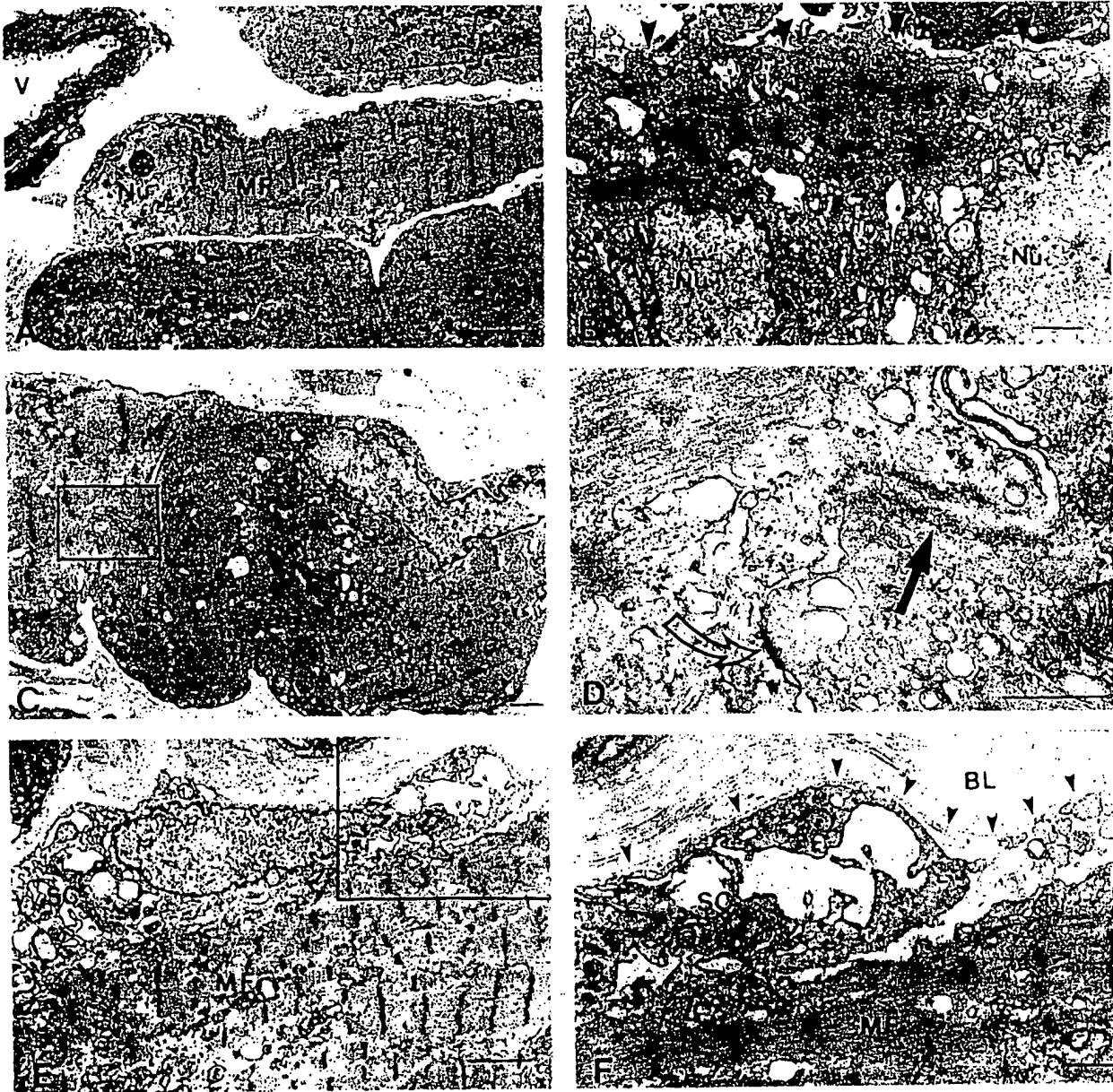


Figure 2. Transmission electron micrographs of 2-wk-old myoblast graft. The graft was placed immediately after cardiac freeze-thaw injury. (A) Low magnification overview showing well differentiated, striated skeletal myofibers (MF) within a collagen-rich matrix. A small venule (V) is shown at the left aspect. Nu, nucleus. Bar, 5 μ m. (B) Moderately differentiated skeletal myofiber containing two nuclei (Nu), a modest complement of myofibrils (mf), and abundant ribosomes and sarcoplasmic reticulum between the nuclei. The sarcolemma is delimited by arrowheads. Bar, 1 μ m. (C) Intercellular junction formation between adjacent myofibers. The two cells have closely apposed and interdigitated membranes. Two electron-dense plaques between the cells are present within the boxed region, suggestive of an adherens type intermediate junction and a gap junction, shown at higher magnification in D. Bar, 1 μ m. (D) Higher magnification of the junctional region boxed in C, showing putative intermediate junction between adjacent myofibers (solid arrow) and gap junction (open arrow). Bar, 0.5 μ m. (E) Skeletal myofiber (MF) with closely apposed mesenchymal cell atop it, suggestive of a satellite cell (SC). The boxed region is shown at higher magnification in F. Bar, 2 μ m. (F) Higher magnification of region boxed in E. The putative satellite cell (SC) and the myofiber (MF) are contained within the same basal lamina compartment (BL, outlined by arrowheads). Although the cell has abundant rough endoplasmic reticulum, its location within the basal lamina of the myofiber and the absence of fibrillar collagen from this space make it unlikely that this is a fibroblast. Bar, 1 μ m.

more rapidly when cells were transplanted into an injury with more advanced healing.

Proliferation of myoblast grafts. To identify cells undergoing DNA synthesis, the thymidine analogue BrdU was admin-

istered for 24 h before time of killing in most groups; animals in the day 1 group received a single pulse of BrdU 1 h before time of killing. Double immunostaining was performed with antibodies to the fast isoform of MHC and to BrdU, to detect

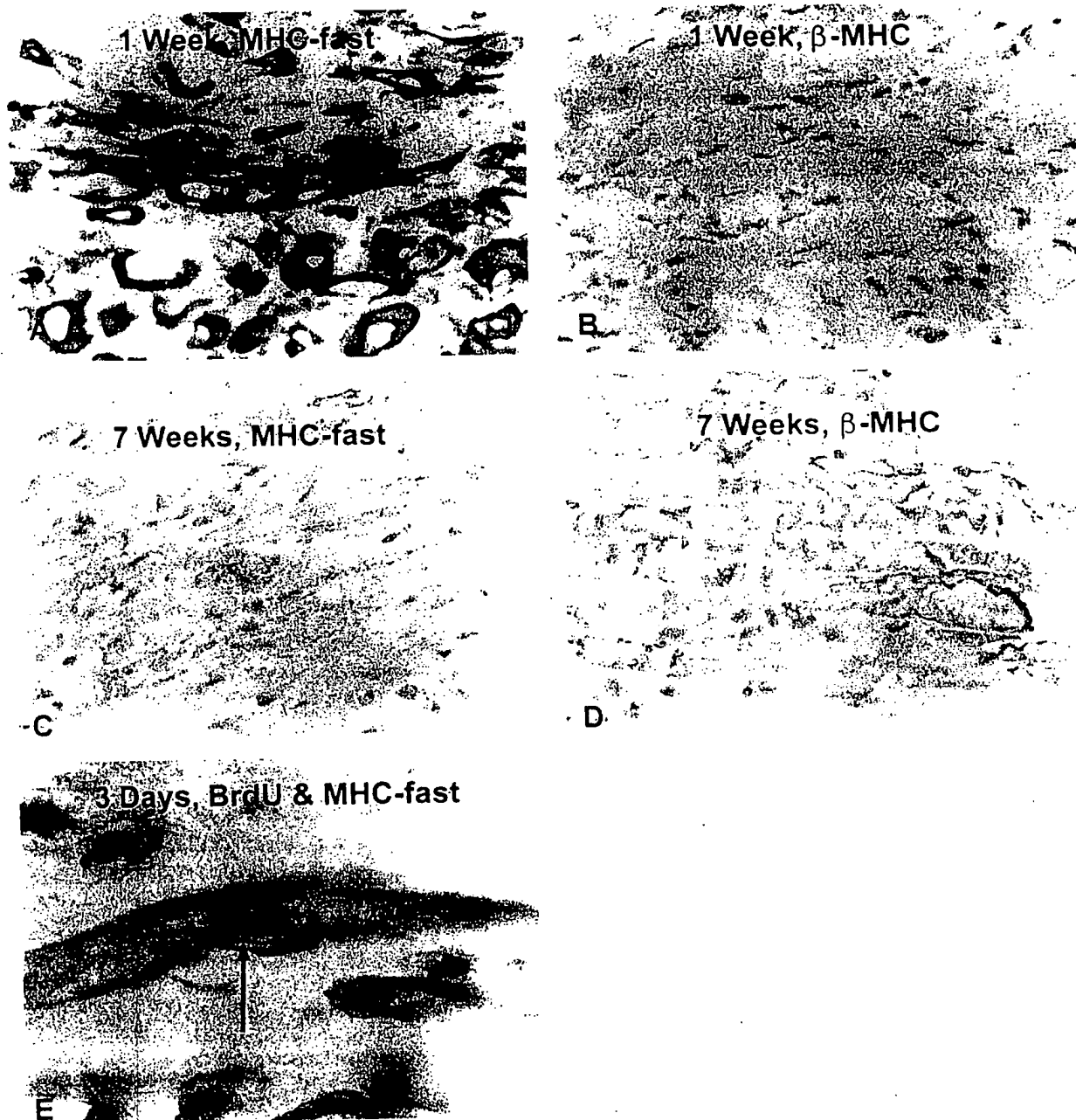


Figure 3. Fiber type conversion and proliferation of engrafted skeletal myoblasts. For the fiber typing experiments, rat hearts were injured by freeze-thaw and the lesions were allowed to heal for 1 wk. Syngeneic skeletal myoblasts were engrafted into the 1-wk-old wounds. For studies of cell proliferation, myoblasts were engrafted immediately after cardiac injury. Rats were killed at the indicated times after transplantation. Antibodies specific to fast twitch (MHC-fast) and slow twitch (β -MHC) fibers were used to define fiber types. Processing for frozen sections in A–D resulted in formation of contraction bands, artifactual clumping of the sarcomeres due to hypercontracture. BrdU was administered 24 h before time of killing to detect DNA synthesis. Double immunostaining for BrdU and MHC was then performed on paraffin sections. Appearance of a BrdU-positive nucleus within a myosin-positive cell indicated the myoblast had replicated and fused into the myotube within the last 24 h. (A) 1-wk graft stained for fast fiber isoforms of MHC. There is intense staining of the engrafted cells, indicating a fast fiber phenotype. Hematoxylin counterstain. $\times 960$. (B) 1-wk graft stained with an antibody to the slow fiber-specific β -MHC. None of the grafted cells express β -MHC at this time, indicating that the cells show no characteristics of slow fibers. Methyl green counterstain. $\times 960$. (C) 7-wk graft stained with an antibody to MHC-fast. There is weak staining compared with the 1-wk graft (A). Methyl green counterstain. $\times 960$. (D) 7-wk graft stained with an antibody to β -MHC. The grafted cells now express β -MHC, indicating that they are acquiring a slow fiber phenotype (compare with B). Methyl green counterstain. $\times 960$. (E) 3-d graft doubly stained for BrdU (purple) and MHC-fast (brown). One nucleus within the myotube stains purple (arrow), indicating it has undergone DNA replication before fusion into the myotube. The remaining nuclei in the myotube do not contain BrdU and pick up the red counterstain. Numerous myosin-negative cells in the surrounding wound tissue also stain positively for BrdU. Nuclear fast red counterstain. $\times 2,400$.

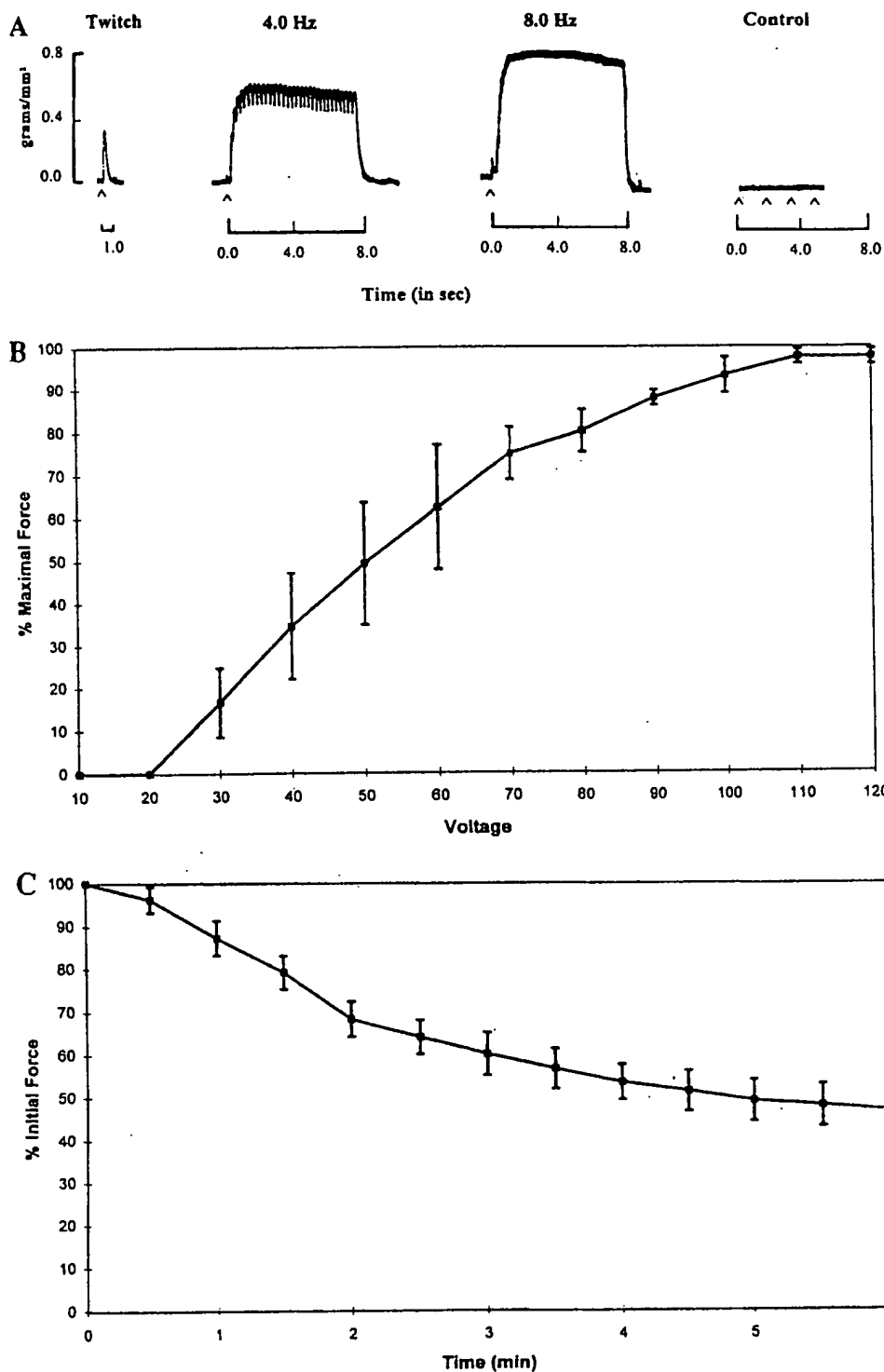


Figure 4. Contractile function of myoblast grafts *ex vivo*. Wound strips from injured hearts receiving either skeletal myoblasts or a sham saline injection were excised at 2 wk. Wounds were mounted on an isometric tension myograph in oxygenated buffer and electrically stimulated. The carats indicate the onset of electrical stimulation. Force has been normalized to cross-sectional area. (A) The first panel shows individual muscle twitch in a myoblast-injected wound. Note the rapid contraction and relaxation rates. The second panel shows that individual twitches began to superimpose with a stimulation frequency of 4 Hz, with a resulting potentiation in developed tension. The third panel shows that tetanus was induced with stimuli ≥ 7 Hz. Note the further increase in tension compared with the 4-Hz stimulation. Time to peak force in this preparation was ~ 1 s, faster than was typically observed for the overall group. The fourth panel shows that no tension was developed at any voltage in a sham-injected wound. This tracing is representative of six wound strips from three sham-injected hearts. (B) Force-voltage relationship. Developed tension for individual twitches increased as stimuli increased from 30 to 100 V, indicating recruitment of additional myofibers. Data have been normalized to maximal developed tension and are presented as mean \pm SEM of eight wound strips from six hearts. (C) Fatigue test. Wounds containing myoblast grafts were subjected to a cardiac-like duty cycle, consisting of repeated episodes of 0.33 s of tetanus/0.67 s of rest, to mimic a heart rate of 60 beats/min. There was a 53% decrease in developed tension at the end of the 6-min test. Note that most of the diminution in force occurred during the first 3 min. Data represent mean \pm SEM of seven wound strips from five hearts.

myoblasts which had proliferated and subsequently differentiated. In the day 1 grafts, proliferating cells were present within the necrotic lesion, which could have represented either graft cells or macrophages. As mentioned above, none of the cells

expressed MHC at this time, so it was not possible to determine which among these were myoblasts (versus transplanted fibroblasts or host macrophages). In the day 3 grafts, occasional BrdU-positive nuclei were identified within myosin-pos-

itive cells (Fig. 3 E). We observed a total of 12 such nuclei in three hearts. No attempt was made to quantify this low rate, but it was certainly $< 1\%$ of total nuclei in myosin-positive cells. Virtually no BrdU-positive nuclei were seen in myosin-positive cells at 1, 2, or 7 wk after transplantation (not shown). We conclude that myoblast proliferation occurs for at least 3 d after grafting, but by 1 wk virtually all cells have ceased replicating.

Contractile function of myoblast grafts. The contractile properties of 2-wk-old myoblast grafts were determined by attaching isolated wound strips to a tension myograph *ex vivo*. Virtually no spontaneous mechanical activity was detected, consistent with the paucity of cardiomyocytes histologically. Electrical stimulation caused muscle twitches in six of eight myoblast-engrafted hearts (Fig. 4 A, *first panel*); strips from the remaining two hearts may have been damaged during sample preparation, since skeletal muscle was present histologically. The grafts showed a stepwise increase in tension development as voltage was increased from 30 to ~ 100 V with a plateau thereafter (Fig. 4 B). This indicates that increasing voltage recruited additional myofibers to contract, implying that the graft myofibers are electrically insulated from one another. It should be noted that cardiac muscle does not increase contractile force with increasing voltage, since cardiocytes are coupled electrically via gap junctions.

Next, force-frequency relationships were determined. Using 120% of the voltage required for maximal tension development, the frequency of stimulation was increased incrementally from 0.5 to 10 Hz. Twitches began to superimpose at frequencies of 3–4 Hz, with a resulting increase in total developed tension (Fig. 4 A, *second panel*). Fully fused tetani were produced with 6–7 Hz stimulation (Fig. 4 A, *third panel*). Peak force during tetanus was 1.98 ± 0.45 grams (mean SEM); after normalization to cross-sectional area the peak force was 0.72 ± 0.14 grams/mm². The time to peak tetanic force averaged 2.3 ± 0.3 s, although 90% of peak force was typically generated within 1.5 s. The time to half-relaxation after tetanus was 240 ± 17 ms. It should be stressed that tetanus cannot be induced in cardiac muscle, due to the long refractory period of cardiocytes.

Finally, a fatigue test was performed to test the response of this muscle to a cardiac-like work load. The grafts were subjected to a duty cycle consisting of repeated 0.33 s of tetanic stimuli followed by 0.67 s of rest, mimicking a heart rate of 60 beats/min. The grafts showed a 32% decline in developed tension by 2 min and a 53% decline by the end of the 6-min test period (Fig. 4 C). No contractile activity could be elicited from six of seven wound strips from three injured hearts which received a sham injection of saline instead of myoblasts (Fig. 4 A, *fourth panel*). In one sham heart an adhesion had developed between the heart and chest wall, resulting in a small amount of intercostal muscle adhering to one of the two wound strips. In this preparation we detected a peak force of 0.04 grams/mm², $< 2\%$ of what was present in the myoblast-engrafted hearts.

Thus, the skeletal muscle grafts could be stimulated to contract *ex vivo* and could sustain a cardiac-like duty cycle over a 6-min test period. Furthermore, the grafts showed two physiological properties unique to skeletal muscle: recruitment of fibers with increasing voltage and the ability to sustain tetanic contraction. We do not know yet whether the grafts contract *in vivo*.

Discussion

The principal findings of this study are: (a) neonatal skeletal myoblasts can be grafted into an injured heart; (b) the engrafted myoblasts initially proliferate and then begin to form multinucleated myotubes by day 3; (c) the myotubes differentiate into mature myofibers, which initially have a phenotype similar to fast twitch muscle; (d) the myofibers develop characteristics of slow twitch muscle as the wound heals; (e) the new muscle may form satellite stem cells; and (f) the new muscle can be stimulated to contract *ex vivo*.

Strategies for muscle regeneration after myocardial injury. In principal, there are at least three strategies to induce muscle regeneration after myocardial infarction. First, the surrounding cardiac myocytes could be stimulated to migrate into the wound and proliferate to repair the defect. There is evidence that a limited amount of cell replication by adult cardiocytes occurs naturally after myocardial infarction in humans (17) and in rats (18, 19), but the response is clearly not adequate to repair the defect. The factors responsible for cell cycle arrest in cardiocytes are not well enough defined at present to begin exploring this as a therapy. (The interested reader is referred to references 20–23 for further information on this topic.)

A second strategy is to induce the cells of cardiac granulation tissue (the fibroblast-rich tissue of wound repair) to differentiate into muscle rather than forming a scar. There is not enough known about cardiac differentiation at present to attempt formation of new myocardium. However, much more is known about skeletal muscle determination. The discovery of myogenic determination genes (24, 25) has made it possible to induce a wide range of cultured cell types to differentiate into skeletal muscle. Recent studies from our group (26) and others (27) have shown that cells in cardiac granulation tissue can be induced to differentiate into skeletal muscle by transfection with the prototype myogenic determination gene, MyoD. In these early experiments, however, the frequency of muscle differentiation has been low after MyoD gene transfer. Until the frequency of myogenic conversion can be increased, it will be difficult to investigate the functional properties of the MyoD-induced skeletal muscle.

The third strategy for muscular repair of infarcts is to transplant either skeletal or cardiac myoblasts into the injured region. Studies by Koh et al. (3) and Soonpaa et al. (2) have demonstrated that fetal cardiocytes will form intercalated discs with host cardiocytes, including gap junctions and adherens junctions, when transplanted into normal hearts. No proliferation was detected in the grafted cardiocytes. Less information is available on grafting of cardiocytes into injured hearts. Our group (28) and others (29, 30) have preliminary data showing that neonatal rat or fetal human cardiocytes can be transplanted successfully into injured rat hearts. To our knowledge there is no information regarding proliferation of these grafts, nor are any functional data available. As discussed above, the principal limitation to this approach is the inability to induce cardiocytes to proliferate in culture. Until cardiocytes can replicate *in vitro*, or proliferation-competent cells can be induced reliably to differentiate into cardiocytes, cardiocyte grafting will not be feasible in humans.

In contrast to cardiocytes, proliferating skeletal muscle precursors are readily available, either as primary myoblasts in developing muscle or as satellite cells from quiescent muscle. In this study six rat pups yielded the myoblasts implanted into

27 injured hearts. In addition to their growth in culture, the myoblasts proliferated *in vivo* for several days after transplantation (Fig. 3 E). These properties have led us and several other groups to explore skeletal muscle grafting for cardiac repair. Koh et al. (7) demonstrated that the myogenic cell line C2C12 could be transplanted into the hearts of normal syngeneic mice, where the cells fused to form multinucleated myofibers. The same group also demonstrated that C2C12 cells stably transfected with a plasmid encoding active TGF- β could induce angiogenesis around the graft site (31). No coupling between the host cardiocytes and the grafted skeletal muscle was observed in either experiment.

Chiu et al. (8, 9) transplanted autologous satellite cells into cardiac freeze-thaw lesions in dogs. Comparable with our study, they also found that the grafts formed muscle cells within the healing lesion. In distinction to the current study, however, they hypothesized that their grafted skeletal muscle cells differentiated into cardiac muscle, via "milieu-dependent effects." The evidence for a cardiac phenotype was that some cells within the grafts had central rather than peripheral nuclei, and some cells contained refractile transverse structures under light microscopy interpreted to be intercalated discs. Although we observed some myofibers with persistent central nuclei in this study, as well as rare cells showing intermediate and gap junctions (Fig. 2, C and D), no intercalated discs were present by electron microscopy. More importantly, the grafted cells expressed skeletal muscle-specific proteins and failed to express the cardiac-specific isoform MHC- α up to 3 mo after transplantation. Thus, there clearly was no cardiac differentiation in this study.

Conversion of grafts from fast to slow twitch muscle. Although the skeletal muscle grafts expressed the fast fiber isoform of MHC at 1 and 2 wk, they expressed β -MHC, a marker for slow twitch fibers, at 7 wk and 3 mo. This indicates that the grafts were converting to slow twitch fibers. Conversion was apparently more rapid when the myoblasts were injected into wounds where healing had been allowed to progress for 1 wk, as opposed to immediately after injury. In the delayed transplantation model the grafts expressed β -MHC at 2 wk, while in the immediate transplantation model this protein was not detected until 7 wk. It is possible that the growth factors and cytokines present in the early wound delay myoblast differentiation and subsequent fiber type conversion.

Slow fibers exhibit several important differences from fast fibers, including a slower shortening velocity, use of oxidative phosphorylation for ATP production, a higher mitochondrial content, a higher myoglobin content, and a much greater resistance to fatigue (16, 32). An interesting parallel is that the latissimus dorsi muscle also undergoes fiber type switching when it is conditioned for dynamic cardiomyoplasty. Cardiomyoplasty is an experimental therapy for heart failure, where skeletal muscle is wrapped around the heart to serve as a ventricular assist device (33). Untrained latissimus dorsi is a mixed fiber type muscle which fatigues rapidly with repeated stimulation. When conditioned by repeated electrical stimulation for 6 wk before surgery, however, the latissimus dorsi converts entirely to slow twitch fibers and becomes fatigue resistant (6). Only the conditioned, slow twitch muscle is able to assist cardiac function. This parallel suggests the intriguing possibility that repeated electromechanical stimulation leads to activation of the slow fiber phenotype. Since we did not test whether the environment of the heart contributed to fiber type conversion,

additional experiments will be required to determine the mechanism. The fact that the grafts differentiated into slow twitch fibers suggests that they may be suited to perform a cardiac type work load.

Will skeletal muscle transplantation augment cardiac function? This study definitively showed that myoblast grafting can generate new contractile tissue. The skeletal muscle grafts exhibited characteristic twitches when stimulated *ex vivo* (Fig. 4 A) and showed recruitment of contractile units with increasing voltage (Fig. 4 B). Furthermore, tetanus could be induced with rapid stimulation (Fig. 4 A, second and third panels), and the grafts could perform a cardiac-like duty cycle for 6 min (Fig. 4 C). Peak force during tetanus averaged 0.7 ± 0.1 grams/mm². Since the wound strips contained < 50% of the myofiber content of normal muscle, due to inclusion of scar tissue, the force can be normalized to at least 1.4 grams/mm² muscle. Adult mammalian muscle can generate 15–35 grams/mm² force at tetanus, depending on fiber type (14, 34). Thus, the 2-wk grafts generated ~ 4–10% of the predicted force for mature skeletal muscle. Several factors may cause a lower than predicted force, including the relative immaturity of the 2-wk myofibers, stretching of the immature extracellular matrix, poor cell matrix attachments, or misalignment of some fibers relative to the axis of the wound strip.

Although preliminary, these results are encouraging and suggest that more detailed studies of contractile function are warranted in skeletal myoblast-engrafted hearts. A critical question is whether the skeletal muscle grafts contract *in vivo*. To provide coordinated mechanical assistance, the grafted cells ideally should form electrical and mechanical junctions with the host myocardium. In our grafts the skeletal muscle cells were insulated from the remaining myocardium by scar tissue, so there was no opportunity for myofiber–cardiocyte coupling to occur. Koh et al. (7) transplanted C2C12 myoblasts into normal mouse hearts and observed no cell junctions between grafted myofibers and host cardiocytes by electron microscopy. Although proliferating myoblasts have been reported to synthesize both gap junction proteins (35) and N-cadherin (36, 37), these proteins are typically absent from adult skeletal myofibers. By electron microscopy we observed evidence both for intermediate and gap junction formation between skeletal myofibers 2 wk after grafting (Fig. 2, C and D). This finding was infrequent, however, and it is unknown whether such junctions would persist in longer term grafts. If skeletal muscle will not couple spontaneously with cardiac muscle, it is possible that such junctions could be induced by stably transfecting skeletal muscle cells with genes for cardiac junctional proteins. Another possibility is that skeletal muscle grafts could be electrically paced in synchrony with the cardiac cycle. Pacing would require sufficient voltage to activate all of the fibers, and currently it is unknown whether this would have a deleterious effect on the surrounding myocardium.

In the uninjured heart there is a complex fiber geometry, where the outer fibers run in the long axis, the midwall fibers run in the short axis, and the inner fibers again run in the long axis. This geometry is established during embryogenesis and is thought to be important for mechanical efficiency. In this study, the grafted myofibers were predominantly aligned with the short (transverse) axis of the heart. Alignment was noted as early as day 3, when myotube formation was prominent (Fig. 1 C). This is the same orientation that wound fibroblasts and collagen fibers acquire during wound healing, and it seems

likely that all are aligned by local mechanical forces. It is not known whether alignment with the heart's short axis will influence the ability of these myofibers to restore mechanical function after injury.

There are two aspects of skeletal muscle which theoretically could make it superior to cardiac muscle for infarct repair. First, skeletal muscle is much more resistant to ischemia than cardiac muscle. Skeletal muscle can withstand many hours of severe ischemia without becoming irreversibly injured, whereas in myocardium irreversible injury begins within 20 min (38). A second difference is that skeletal myoblast grafts might establish satellite cells. Satellite cells are the resident stem cells in skeletal muscle and proliferate in response to injury. Once activated, satellite cells can fuse with damaged myofibers or establish new myofibers to replace those lost to necrosis. We observed cells within 2-wk grafts which were morphologically consistent with satellite cells by electron microscopy (Fig. 3, E and F). Thus, it is possible that infarcts repaired with skeletal myoblasts might become more resistant to a subsequent episode of ischemia or might be able to replace myofibers damaged by ischemia.

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EVIDENCE APPENDIX

ITEM NO. 10

**July 1, 2005 publication in The Journal of Invasive Cardiology,
Vol. 17, entitled “Progenitor Cell Transplantation and
Function following Myocardial Infarction” (author unknown)
cited by the Examiner in the October 2, 2008 Office Action**

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Progenitor Cell Transplantation and Function following
Myocardial Infarction

VOLUME: 17 PUBLICATION DATE: Jul 01 2005

Issue Number:

7

William O'Neill: I have a couple of questions for both of you. First, we are essentially talking about adult, autologous stem cells. As you all know, there is enormous controversy in the U.S. about the use of embryonic stem cells which we are currently prohibited from using. From a dispassionate, basic scientific approach, do you think there's an advantage to using embryonic stem cells, or will adult stem cells be sufficient in terms of their ability to replicate and cause myogenesis?

Peter Gonschior: After examining the data from experiences in other fields with vascular cells and their biological functions, I don't think there is a need for embryonic stem cells. The bone marrow cells appear to be very robust; even the non-randomized European trial data show that with a not-so-profound approach, it is possible to obtain positive effects. Due to the political scenery in Europe, I don't think it would be possible to conduct large clinical trials using embryonic stem cells.

Sigrid Nikol: I think we should first try to optimize the strategies using autologous cells due to the ethical concerns. Due to these restrictions, there is not currently much proof available that embryonic stem cell therapy is that much better or even less risky. We know that these cells can differentiate, which means that they could differentiate into undesired cells as well. Thus, given the legal restrictions at present, we should stick with what we can do and try to make the best of it.

William O'Neill: Another key issue that arises from a clinical standpoint is that of direct injection versus intra-arterial or intravenous infusion. Do we know yet which would be the most efficient way — if the appropriate cell were to be found — to deliver the cells for the most efficient myogenesis?

Peter Gonschior: Sigrid and I have a lot of experience with local drug delivery. Efficiency of local drug delivery is in fact very low. It would be very smart to just inject the cells intravenously. But one critical issue with all of these data is that delivery efficiency, whether systemic or local, is very low. In fact, it turned out that endocardial delivery of these cells was the most efficient, resulting in the largest number of injected cells — up to about 30 million.

William O'Neill: The problem I have is that the stem cells are very sensitive to hypoxia; they need an oxygenated environment in order to thrive. Thus, if these cells are injected into a core infarct area, it will likely be hypoperfused and the ambient oxygen tension in that area may not be sufficient to support those cells. Sigrid, your experience involved a permanent ligation, right?

Sigrid Nikol: Actually, there were two sets of animals, but I only showed you those with the permanent ligation because there are currently more data on them. We also have a reperfusion model from 30 minutes after opening up the vessel.

William O'Neill: Did you find in the reperfusion model that there was any more myogenesis efficiency?

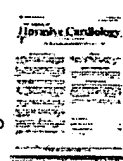
Sigrid Nikol: We perform stem cell therapy via bone marrow stimulation (not cell injection). At the moment, we do not have sufficient data on the reperfusion model. The problem is that when genes or cells are injected into the vessels, why should they pass the endothelium and migrate into the tissue where they are needed? It is very unlikely to happen, unless there is injured tissue. And with the venous application, there are the first-pass effects in the lungs and liver on top of it. These findings are not very convincing in my opinion, including the data from the Strauer group which lacked a truly randomized control group. The problem with intramyocardial injections into infarcted areas is a real one, as you stated, in that there is no blood/oxygen supply and potentially arrhythmic foci are created. Also, there may not be a homogeneous distribution of cells — an issue that has already been discussed with regard to gene therapy for the myocardium. Specifically, the question has been raised regarding how homogeneous gene therapy in the myocardium can be achieved without creating arrhythmias. With stem cells, this may be an even more important issue, as has been demonstrated in the work by Menasche et al.

William O'Neill: It seems to me that a highway is needed to get the cells to the tissue, and that's why we have been considering autotransfusion or direct intracoronary infusion of the agents, because in the reperfusion model, at least if there's an intact vascular structure, then the environment will be ambient and oxygenated until the cells can engraft. But my question is: Why would those cells stay at that target site? Also, we are lacking basic scientific understanding of the signals that allow the stem cells to home in on that particular tissue. Since stem cells could potentially be coming in from the systemic circulation, we need to come up with a homing material that could be injected into the infarcted area and would allow the stem cells to accumulate there. We are moving forward in this field.

At the 2003 AHA meeting, data from the late-breaking BOOST trial were presented. It was, I think, a very well-conducted randomized trial involving 60 patients with placebo versus bone marrow infusion arms. The results showed a statistically significant increase in ejection fractions in the active treatment group compared with the placebo group. TOPCARE was a terrific study, but it was a sort of historical control study. Thus, we are beginning to accumulate data that suggest there is some merit to this approach. Let me turn this question over now to the other panelists. Matt Pollman, from Guidant Corporation, is on the panel. Matt, is there business potential in this field?

Matt Pollman: That's a great question, Bill. As a clinician and a scientist, this area is extremely provocative and intriguing, particularly the data from the BOOST and TOPCARE myocardial infarction trials. There is an aggressive movement to push these trials out into more randomized, controlled, multi-center arenas. The issue for a company such as Guidant is what we can bring to the table and whether there is business potential in this area. Confidentiality, of course, prevents me from saying too much here, but there certainly appear to be business opportunities for companies like ours. We believe that it will require a very safe delivery system that can be applied to a wide range of patients. The cells clearly need to be delivered at least very close to the target site in order to successfully do their job. In that light, an intracoronary infusion strategy raises questions about how to allow enough time for the cells to adhere, to translocate, enter the tissue and do their job. There is room for improvement in the delivery systems, and that's what Guidant is looking into. The TOPCARE acute myocardial infarction study headed up by Andreas Zeiher in Frankfurt uses a simple syringe injection system loaded with 10 cc of bone marrow, 3 cc of which is applied to the coronary arteries. The patients are then allowed to be in an unperfused state for 3 minutes to allow the cells to do their job. Often, the limiting factor is that the patients complain of chest pain during the unperfused state. Thus, there are opportunities to improve the current infusion protocol.

William O'Neill: We have been looking at it in a two-prong fashion, with a lot of interest in basic science on one end, and in clinical trials on the other end. As for the United States, if bone marrow is taken from a patient and then re-injected, the FDA considers it non-homologous use, even though it is not a drug or a commercial compound. The U.S. FDA regulations on stem cell use do not cover autologous use of these cells. Unfortunately, thus, this entire field has become embroiled in the embryonic stem cell and abortion debates. As a result, the FDA is incredibly cautious about allowing embryonic stem cell use. The FDA regulations state that if the stem cells are taken and not manipulated, and then re-injected, it falls outside of the FDA's authority. At the 2003 TCT meeting, the FDA representative specifically stated that if any

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EVIDENCE APPENDIX

ITEM NO. 11

**Strauer et al. 2005 publication in Circulation entitled,
“Regeneration of Human Infarcted Heart Muscle by
Intracoronary Autologous Bone Marrow Cell Transplantation
in Chronic Coronary Artery Disease” cited by Applicant as
Exhibit D in the Amendment filed November 21, 2005**

Regeneration of Human Infarcted Heart Muscle by Intracoronary Autologous Bone Marrow Cell Transplantation in Chronic Coronary Artery Disease

The IACT Study

Bodo E. Strauer, MD,* Michael Brehm, MD,* Tobias Zeus, MD,* Thomas Bartsch, MD,* Christina Schannwell, MD,* Christine Antke, MD,† Rüdiger V. Sorg, PhD,‡ Gesine Kögler, PhD,‡ Peter Wernet, MD,‡ Hans-Wilhelm Müller, MD,† Matthias Köstering, MD*

Düsseldorf, Germany

OBJECTIVES	Stem cell therapy may be useful in chronic myocardial infarction (MI); this is conceivable, but not yet demonstrated in humans.
BACKGROUND	After acute MI, bone marrow-derived cells improve cardiac function.
METHODS	We treated 18 consecutive patients with chronic MI (5 months to 8.5 years old) by the intracoronary transplantation of autologous bone marrow mononuclear cells and compared them with a representative control group without cell therapy.
RESULTS	After three months, in the transplantation group, infarct size was reduced by 30% and global left ventricular ejection fraction (+15%) and infarction wall movement velocity (+57%) increased significantly, whereas in the control group no significant changes were observed in infarct size, left ventricular ejection fraction, or wall movement velocity of infarcted area. Percutaneous transluminal coronary angioplasty alone had no effect on left ventricular function. After bone marrow cell transplantation, there was an improvement of maximum oxygen uptake (VO_{2max} , +11%) and of regional ^{18}F -fluor-desoxy-glucose uptake into infarct tissue (+15%).
CONCLUSIONS	These results demonstrate that functional and metabolic regeneration of infarcted and chronically avital tissue can be realized in humans by bone marrow mononuclear cell transplantation. (J Am Coll Cardiol 2005;46:1651-8) © 2005 by the American College of Cardiology Foundation

Cardiac performance after myocardial infarction (MI) is compromised by ventricular remodeling, which represents a major cause of late infarct-related chronic heart failure and death (1,2). Although conventional drug therapy (e.g., with beta-receptor blockers and/or angiotensin-converting enzyme inhibitors) may delay remodeling, there is no basic

See page 1659

therapeutic regimen available for preventing or even reversing this process. By the use of interventional therapeutics (percutaneous transluminal coronary angioplasty [PTCA], stent), recanalization of the occluded infarct-related artery is possible, thereby improving or normalizing coronary blood flow. However, despite sufficient reperfusion of infarcted tissue, the viability of the infarcted myocardium cannot, or can only insufficiently, be improved in most of these patients (3). Therefore, catheter-based therapy of acute MI is useful for vascular recanalization, but the second and crucial step,

the regeneration of necrotic heart muscle, is not realized by this vascular procedure alone.

Experimental (4) and clinical (5,6) studies have shown recently for the first time that bone marrow mononuclear cells (BMCs) may regenerate damaged myocardium in acute MI in humans. Because the regenerative potential of bone marrow-derived cells ought also to be expected to exist in chronically ischemic heart disease as well (7-12), we have assembled in an ongoing clinical investigation 18 patients with chronic MI to prove this new therapeutic possibility.

METHODS

Study population. All 18 patients (49 ± 11 years) were men and were recruited consecutively from January 2003 until March 2004. They had had transmural MI 27 ± 31 months before, at which point all infarcts had been treated acutely by PTCA and/or stent implantation (Table 1, Fig. 1).

The inclusion criteria were age <70 years, one-vessel disease with an open infarct-related artery at the time of stem cell therapy, sinus rhythm, a clear-cut demarcation of the ventriculographic infarct area, and no coronary bypass surgery. General exclusion criteria were severe comorbidity and alcohol or drug dependency. Although chronically infarcted myocardium usually does not regenerate sponta-

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Abbreviations and Acronyms

BMC	= bone marrow mononuclear cell
CPK	= creatine phosphokinase
ECG	= electrocardiogram
LV	= left ventricular
MI	= myocardial infarction
PET	= positron emission tomography
PTCA	= percutaneous transluminal coronary angioplasty
Tx group	= transplantation group

neously, for comparison a control group, parallel to the recruitment of the stem cell transplantation group (Tx group), was recruited and analyzed, meeting the same inclusion criteria as the stem-cell group. The recruitment of patients was performed according to a randomization procedure in which all patients of the entire chronic infarction group were distributed to the treatment group, where they agreed with all the therapeutic regimen. Alternatively, all patients of the chronic infarction group who refused the therapeutic regimen (bone marrow puncture and aspiration, intracoronary cell transplantation, and another cardiac catheterization) were allocated to the control group. All medications with angiotensin-converting enzyme inhibitors and with beta receptor blockers were maintained constant during the study period.

The cell-treated patients had stable ventricular dynamics for infarct size, ejection fraction, and wall movement velocity of infarcted area at least 9 ± 6 months before cell transplantation. Infarct size at the time of cell therapy showed an amount of $27 \pm 8\%$ of the circumference of the left ventricle (LV), determined by ventriculography.

Preparation of BMCs. One day before cell therapy, bone marrow was taken (80 ml from the iliac crest) and mono-

nuclear cells were isolated and identified including CD34-positive cells, AC133-positive cells and CD45/CD14 negative cells (6). The cells were isolated under good manufacturing practice conditions by Ficoll density separation on Lymphocyte Separation Medium (Bio Whittaker, Walkersville, Maryland), before the residual erythrocytes were lysed with H_2O . For overnight cultivation, 1×10^6 BMCs/ml were placed in Teflon bags (Vuelife, Cell Genix, Gaithersburg, Maryland) and cultivated in X-Vivo 15 Medium (Bio Whittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed three times with heparinized saline before final resuspension in heparinized saline. Viability was $93 \pm 3\%$. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. These cells were used for therapy. All microbiologic tests of the clinically used cell preparations proved negative. All patients received extensive information about the procedure, which was approved by the ethical committee of our university, and all gave written informed consent.

Administration of BMCs. Following assessment of baseline examinations (coronary angiography, left ventriculography, spiroergometry, ^{99m}Tc -tetrofosmin single-photon emission computed tomography (SPECT) and ^{18}F -fluor-deoxy-glucose (^{18}F -FDG) positron emission tomography (PET), cell transplantation was performed via the intracoronary administration route (6,13) using four to six fractional infusions parallel to balloon inflation over 2 to 4 min of 3 to 5 ml of cell suspension, each containing 15 to 22×10^6 mononuclear cells. All cells were infused directly into the infarcted zone through the infarct-related artery via an angioplasty balloon catheter, which was inflated at a low pressure (2 to 4 atm) and was located within

Table 1. Demographic Data of Intracoronary Bone Marrow Stem Cell Transplantation Group and Control Group

Characteristics	Tx Group	Control Group	p
No. of patients	18	18	
Age, yrs	49 ± 11	52 ± 10	NS
Transmural myocardial infarction, months before Tx	27 ± 31	30 ± 34	NS
Coronary angiography			
LAD/LCX/RCA as affected vessel	16/0/2	10/3/5	
No. of patients with stent implantation	16	17	NS
Risk factors			
Diabetes mellitus, %	16	11	NS
Positive family history, %	44	33	NS
Smoker and ex-smoker, %	67	56	NS
Hyperlipoproteinemia, %	89	94	NS
Medication			
Beta-blocker, %	94	89	NS
Angiotensin-converting enzyme inhibitor, %	94	89	NS
Statins, %	94	100	NS
Laboratory parameters			
CPK, U/l	$1,504 \pm 979$	$1,489 \pm 952$	NS
Bone marrow mononuclear cells, n ($10^6 \times$)	90		

Values are mean \pm SD or number of patients.

CPK = creatine phosphokinase; LAD = left anterior descending coronary artery; LCX = left circumflex coronary artery; RCA = right coronary artery; Tx = intracoronary bone marrow stem cell transplantation.

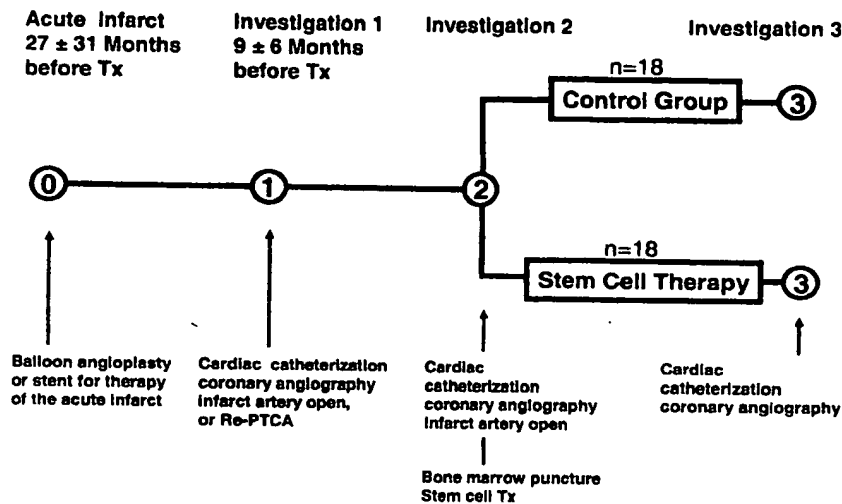


Figure 1. Diagrammatic representation of the algorithm of intracoronary stem cell therapy (Tx) in chronic ischemic heart disease after myocardial infarction. The infarcts occurred 27 ± 31 months before Tx. All infarct patients were treated with percutaneous transluminal coronary angioplasty (PTCA) or with stent implantation. 9 ± 6 months before (investigation 1) coronary angiography (including quantitative left ventriculography) was performed. If re-stenosis was present, re-PTCA was made. Investigation 2 embraces all patients for the evaluation of coronary morphology after PTCA/stent. Only patients with an open infarct-related artery were included in both groups. Patients who agreed to Tx received within 10 days after investigation 2 bone marrow punctures and Tx by the intracoronary administration route and had altogether five invasive investigations, including two for therapeutic reasons (nos. 0 and 1). Patients who were not eligible for Tx (disagreement with bone marrow puncture and with subsequent Tx) served as a control group. Investigation 3 represents all follow-up measurements 3 months after Tx (Tx patients) or after investigation 2 for control group patients.

the previously stented coronary segments. This prevented backflow of cells and produced stop flow beyond the site of balloon inflation to facilitate high-pressure infiltration of cells into the infarcted zone. Prolonged contact time for cellular migration was also enabled. Three months after catheter-guided cell transplantation, all functional tests were repeated, including coronary angiography and left ventriculography. There were no procedural or cell-induced complications, and there were no side effects in any patient.

Spiroergometry. Aerobic exercise capacity was examined before (<10 days) intracoronary cell transplantation and three months later during follow-up. All patients ($n = 18$) were subjected to initial bicycle spiroergometry to assess their functional fitness and to determine the limit of safe intensity of exercise. We chose a protocol with an intensified workload up to the symptom-limited maximum (basic load of 50 W, intensification at 25 W, 2-min duration of each workload step). We determined the anaerobic threshold for prescribing a suitable load intensity. During the whole spiroergometry, monitoring by a 12-lead electrocardiogram (ECG) was carried out. The exercise capacity was assessed on the basis of maximum load levels expressed in watts (W_{max}) and maximum peak oxygen uptake (VO_{2max}).

Coronary angiography and left ventriculography. Coronary angiography and biplane left ventriculography were performed 9 ± 6 months before cell transplantation and also a second time, within 10 days, immediately before cell therapy. The therapeutic follow-up was three months after the treatment. Thus, stable baseline conditions were documented (coronary vessel involvement, ventricular function, and geometry). Cardiac function was evaluated by left

ventricular (LV) ejection fraction and by auxotonic myocardial contractility index, evaluated by the wall movement velocity of the infarcted area. The infarct size was calculated according to the method of Sheehan (14) by plotting five axes perpendicular to the long axis of the heart in the main akinetic or dyskinetic segment of the ventricular wall. Systolic and diastolic lengths were then measured by two independent observers, and the mean difference was divided by the systolic duration in seconds.

Quantification of coronary stenosis (restenosis). Cinecoronangiograms were obtained during stem cell transplantation and at three months thereafter according to standard acquisition guidelines. The angiograms were evaluated by two independent observers and quantitative analysis was performed (15). Standard morphologic criteria were used to characterize the complexity of baseline lesions. The user-defined reference diameter proximal to the stenosis and the minimal luminal diameter within the culprit of the stenosis were used to calculate the percentage of stenosis. A value of 0 mm was assigned for the minimal luminal diameter in case of total occlusion at baseline or follow-up. Restenosis was defined as $\geq 50\%$ stenosis of the initial target lesion at follow-up. Calculations of restenosis were performed in both groups, with and without stem cell therapy, in the same way, thus enabling evaluation the differential effects of PTCA-guided cell therapy and of PTCA effects alone.

Ventricular function after PTCA in the control group. For the evaluation of a potential effect on the PTCA intervention itself on LV function, all patients in the control group were analyzed with regard to infarct size, ejection fraction, and infarction wall movement velocity.

Table 2. Single Values of Intracoronary Bone Marrow Stem Cell Transplantation Group

Patient Number	Area of Infarction, %*				LV Ejection Fraction, %*				Infarction Wall Movement Velocity, cm/s*			
	Investigation 1		Investigation 2		Investigation 3		Investigation 1		Investigation 2		Investigation 3	
	9 ± 6 Mo Before Tx	9 ± 6 Mo After Tx	<10 Days Before Tx	<10 Days After Tx	9 ± 6 Mo Before Tx	9 ± 6 Mo After Tx	9 ± 6 Mo Before Tx	9 ± 6 Mo After Tx	<10 Days Before Tx	<10 Days After Tx	9 ± 6 Mo Before Tx	9 ± 6 Mo After Tx
1	26	22	26	22	56	55	0.88	0.82	0.77	0.77	0.88	0.82
2	28	26	29	26	45	43	2.06	2.13	1.88	1.88	2.06	2.13
3	16	5	16	5	64	65	1.45	2.10	1.50	1.50	1.45	2.10
4	27	14	25	14	48	50	1.20	2.88	1.25	1.25	1.20	2.88
5	16	11	14	11	66	69	2.25	3.75	2.77	2.77	2.25	3.75
6	16	6	13	6	64	66	1.50	2.55	1.77	1.77	1.50	2.55
7	15	11	18	11	57	55	2.78	3.13	2.65	2.65	2.78	3.13
8	28	20	28	20	43	44	3.15	4.25	3.25	3.25	3.15	4.25
9	27	11	27	11	46	46	1.61	3.30	1.65	1.65	1.61	3.30
10	20	14	17	14	56	58	2.21	3.13	2.45	2.45	2.21	3.13
11	28	17	25	17	42	38	1.91	3.00	1.88	1.88	1.91	3.00
12	33	21	28	21	44	47	2.28	3.50	2.62	2.62	2.28	3.50
13	39	27	37	27	50	51	1.25	4.90	2.50	2.50	1.25	4.90
14	29	33	33	33	62	62	1.20	2.70	1.33	1.33	1.20	2.70
15	37	31	37	31	48	43	1.83	2.50	1.56	1.56	1.83	2.50
16	29	24	29	24	53	54	1.25	3.06	1.06	1.06	1.25	3.06
17		35	41	35		48		3.00	1.66	1.66		3.00
18		25	35	25		45		1.94	0.94	0.94		1.94
Mean	26	19	27	19	53	52	1.80	2.92	1.86	1.86	1.80	2.92
SD	7	9	8	9	8	9	0.63	0.91	0.70	0.70	0.63	0.91

*Calculated from left ventriculography.

LV = left ventricular; Mo = Months; other abbreviations as in Table 1.

Nuclear cardiologic investigations (PET and SPECT). ^{18}F -FDG-positron emission tomography (^{18}F -FDG PET) was performed with a Scanditronix SCX 4096 WB-Scanner (FWHM = 6 mm transaxial, axial field of view = 4.6 cm). Patients received an oral glucose load of 1 g/kg body weight 80 ± 30 min before the intravenous application of ^{18}F -FDG (380 ± 60 MBq). The ^{18}F -FDG was administered at the time of decrease of blood glucose level <130 mg/dl. An initial transmission scan was obtained using a ^{68}Ga -filled pin source to correct the subsequent emission scans for attenuation. The data acquisition was started 45 min after administration of FDG. Image data were recorded with a 256×256 matrix in 3 consecutive bed positions over 15 min per position. The data were reconstructed backprojected with a Hanning filter (5 mm).

$^{99\text{m}}\text{Tc}$ -tetrofosmin SPECT. Sixty minutes after intravenous injection of 600 ± 140 MBq of the perfusion-marker $^{99\text{m}}\text{Tc}$ -tetrofosmin under a "rest" condition, the images were obtained using a SPECT scanner with double-head detector (PRISM 2000, Marconi/Phillips), a low-energy, high-resolution collimator, and a 128×128 matrix. Image data were collected over 360° at 3° every 30 s. The images were reconstructed backprojected with a low-pass filter (order 12, cutoff 0.2).

PET and SPECT evaluation. Normalized values for FDG uptake and perfusion were calculated by comparing regional with maximum tracer uptake on the reconstructed images. We performed a regional analysis of glucose metabolism and perfusion using a set of standardized, individually adjusted circular regions of interest (diameter 18.06 mm, surface 256 mm^2). The reconstructed metabolic and perfusion images were realigned for each patient (MPI-Tool, version 3.0; Advanced Tomo Vision, Erftstadt, Germany) and were resliced according to cardiac axis (short-axis and horizontal and vertical long-axis views). The regions were positioned immediately neighboring, with no overlap, according to an overlay of the co-registered metabolic and perfusion images. The regions covered the infarct lesion as well as normal myocardium. In this way, we generated templates of regions for each patient, which could be used for the evaluation of metabolism and perfusion, before and after BMC transplantation without further modification. According to Segall et al. (16), regions with a normalized FDG uptake $<50\%$ were rated as transmural scar and regions with an uptake of 50% to 60% as non-transmural scar.

Further analysis was restricted to regions with FDG uptake $<60\%$ in the PET scans, pursuant to our intention to focus on the effects of BMC transplantation on scar tissue.

Safety parameters. To assess any inflammatory response and myocardial reaction after cell therapy, white blood cell count, the serum levels of C-reactive protein (CRP) and of creatine phosphokinase (CPK) were determined immediately before as well as after treatment. Additional analysis was done directly after transplantation and three months later: ECG at rest, 24-h Holter ECG, and echocardiography.

Statistical analysis. All data are presented as mean \pm SD. Statistical significance was accepted when $p < 0.05$. Intra-individual comparison of variables of investigation 1 (9 ± 6 months before cell transplantation for Tx group, 9 ± 5 months before investigation 2 for control patients) and investigation 2 (<10 days before cell transplantation for Tx group, no transplantation for control patients) and of variables of investigation 2 and follow-up investigation 3 (3 months after cell therapy for Tx group, 8 ± 5 months after investigation 2 for control patients) was performed using Wilcoxon rank-sum test. The missing values (Table 2) were omitted and not calculated for statistical analysis. The p values (by analysis of variance) have been given for LV ejection fraction, area of infarction, and infarction wall movement velocity. Statistical analysis was performed with SPSS-Windows 10.1 software.

RESULTS

Three months after intracoronary cell therapy, the infarct size was reduced by 30%, whereas the global LV ejection fraction increased by 15% and regional infarct wall movement velocity by 57% (Tables 2 and 3). In parallel, the clinical performance improved (Table 4), as evidenced by a higher work load demonstrated by a 11% increase in maximum oxygen uptake ($\text{VO}_{2\text{max}}$). SPECT investigation presented enhanced tetrofosmin uptake in the infarcted zone by 5%, and PET examination showed enhanced glucose uptake in the infarcted zone by 15%, demonstrating regeneration of formerly avital, chronically infarcted heart muscle (Fig. 2). An unchanged or even impaired LV function was not observed in any patient.

In the control group (18 patients with chronic MI, but without stem cell therapy) no significant changes were observed in infarct size, LV ejection fraction, or wall

Table 3. Cardiac Parameters in the Transplantation Group and in Control Group at the Three Investigation Time Points

	Area of Infarction, %			LV Ejection Fraction, %			Infarction Wall Movement Velocity, cm/s		
	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*	Control Group	Tx Group	p Value*
Investigation 1	25 ± 9	26 ± 7	0.99	53 ± 10	53 ± 8	0.87	1.95 ± 0.66	1.80 ± 0.63	0.57
Investigation 2	27 ± 9	27 ± 8	0.83	51 ± 10	52 ± 9	1.00	1.88 ± 0.76	1.86 ± 0.70	0.94
Investigation 3	26 ± 9	19 ± 9	0.02	52 ± 10	60 ± 7	0.02	1.91 ± 0.79	2.92 ± 0.91	0.001

*Analysis of variance.

Abbreviations as in Table 1.

Table 4. Positron Emission Tomography and Spiroergometry Before and After Stem Cell Therapy in Chronically Infarcted Myocardium

	¹⁸ F-FDG-Positron Emission Tomography			VO _{2max} Spiroergometry	
	FDG Uptake, %	Difference in %		ml/min	Difference in %
Investigation 1	none			none	
Investigation 2	43.8 ± 8.0	>	+ 15	1,602 ± 533	>
Investigation 3	50.5 ± 11.6			1,776 ± 523	
p (Wilcoxon test)	0.012			0.0001	

¹⁸F-FDG = ¹⁸F-fluor-deoxy-glucose; VO_{2max} = maximum oxygen uptake.

movement velocity of the infarcted area (Figs. 3A to 3C). Electrocardiogram at rest and on exercise and 24-h Holter ECG revealed no rhythm disturbances at any time point. Only 1 patient (from 18 cell-treated patients, 6%) developed relevant restenosis due to quantitative angiographic criteria. The restenosis could be treated adequately by stent implantation. The other 17 patients showed good patency rates without restenosis after PCI and cell transplantation. They also revealed no alterations in LV function 8 ± 5 months after PTCA.

There was no inflammatory response or myocardial reaction (white blood cell count, CRP, CPK) after cell therapy, despite a moderate increase in CRP (before cell transplantation 0.58 ± 0.48 mg/dl, after cell transplantation 1.07 ± 0.73 U/l, $p = 0.002$), which is usual after bone marrow puncture and/or cardiac catheterization.

DISCUSSION

The results of these investigations demonstrate, for the first time, that the intracoronary transplantation of autologous bone marrow mononuclear cells may reduce infarct size and improve LV function as well as myocardial glucose uptake in chronic ischemic heart disease attributable to chronic MI (5 months to 8.5 years old). Infarct size decreased in all patients and cardiac performance (ejection fraction, wall movement velocity of infarcted area, maximum oxygen uptake, and exercise tolerance) and myocardial metabolism (FDG-PET) improved, all being between 11% and 57%. Furthermore, it is noteworthy that there were no complications immediately or three months after cell transplantation, especially that there was no cardiac arrhythmia and no signs of cardiac or systemic inflammation were present.

The effects of stem cell transplantation on infarct size, cardiac function, and contractility demonstrate significant improvement of these three parameters in the therapy group (before and after stem cell therapy) as well as in the comparison between the stem cell therapy group and the control group, thus giving evidence for a beneficial therapeutic effect of stem cell therapy on cardiac performance in chronic MI.

Patients in both the stem-cell group and the control group were recruited in parallel to each other and consecutively between January 2003 and March 2004. They all ($n = 36$) fulfilled the same inclusion criteria. Thus, representative patient characteristics were present for the stem cell group ($n = 18$) and the control group ($n = 18$) as well as in comparing both of them. Moreover, two subsequent investigations before stem cell transplantation have been performed for each patient: investigation 1 and 2 demonstrated the stability of LV dynamics before cell therapy (9 months respectively 10 days before transplantation) and investigation 3 compared the effects of stem cell therapy with the control group. The stable hemodynamics during the preceding 9 ± 6 months before stem-cell therapy and the stable hemodynamics within the control group at all three points of investigation underline the significant alterations of the left ventriculography-derived parameters investigated after stem cell transplantation.

The regenerative potential of bone-marrow-derived stem cells may be explained by any of four mechanisms: 1) direct cell differentiation from mononuclear cells to cardiac myocytes (17), 2) cytokine-induced growing and increase of residual viable myocytes, especially within the border zone of the infarcted area (18), 3) stimulation of intrinsic myocardial stem cells (endogenous stem cells) (19,20), and 4)

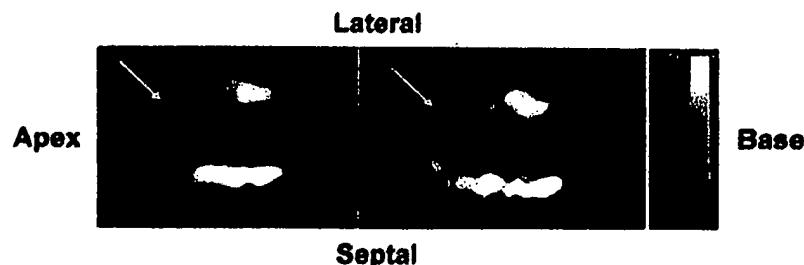


Figure 2. Representative illustration of ¹⁸F-FDG-positron emission tomography (PET) before (above) and 3 months after (below) cell therapy in the transversal (left) and longitudinal (right projection) in a 30-year-old male patient with an 8-month-old anteroapical infarction. Note the restoration of glucose uptake (below) within the infarcted area of the formerly completely avital anteroapical myocardium.

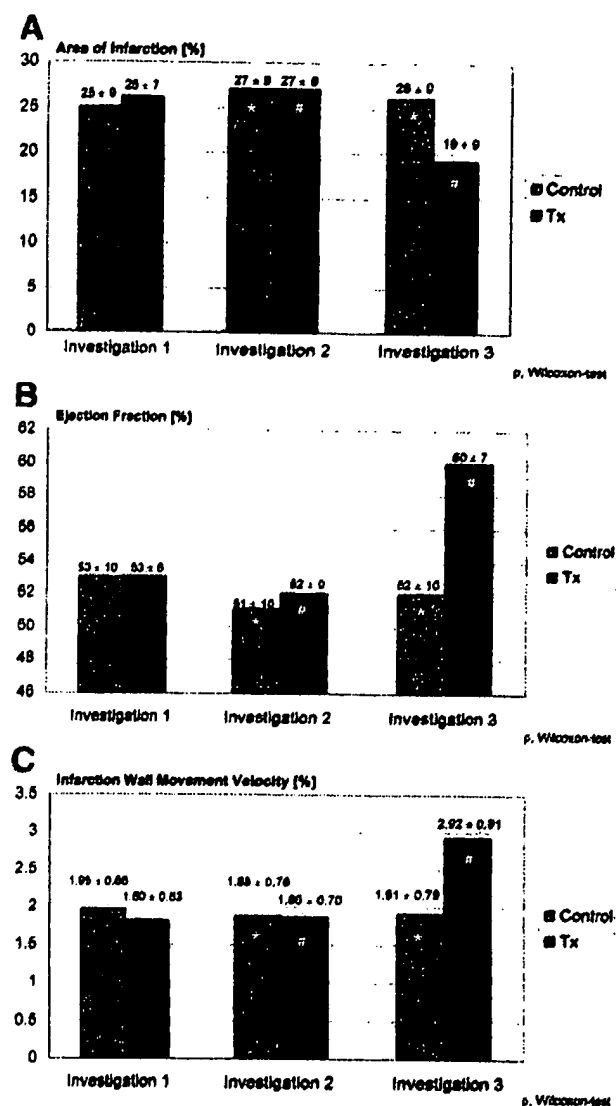


Figure 3. Illustration of the mean values of (A) area of infarction, (B) ejection fraction, and (C) infarction wall movement velocity, determined by quantitative left ventriculography in both groups (control group vs. transplantation [Tx] group) at the point of time: investigations 1, 2, and 3. Comparison of both groups with chronically infarcted myocardium (control group vs. Tx group), *n* = 18 patients. Investigation 1 was 9 ± 6 months before cell transplantation (controls: 9 ± 5 months before percutaneous transluminal coronary angioplasty [PTCA]); investigation 2 within 10 days before cell transplantation (controls: at the time point of PTCA) and investigation 3 was three months after cell transplantation (controls: 8 ± 5 months after PTCA). Note the significant decrease of infarct size and the increase in ejection fraction and in contractility (infarction wall movement velocity) 3 months after cell therapy in comparison with the control group. **p* = not significant (investigation 2 vs. investigation 3); #*p* = 0.001 (investigation 2 vs. investigation 3).

induction of cell fusion between transplanted bone marrow cells and resident myocytes (21–24).

Transdifferentiation has been described by previous investigators (4); however, it has been questioned by recent experimental studies (25). The influence of cytokines has

shown to restore coronary blood vessels and muscle cells after experimental myocardial infarction. This regeneration of blood vessels and muscle cells is most pronounced in the border zone of ischemic and/or infarcted tissue (26), demonstrating an enhancement of mitotic cells and cell cycles up four-fold, when compared to areas remote from the necrotic myocardium. Moreover, mononuclear bone marrow stem cells contain a lot of cytokines (VEGF, insulin-like growth factor, platelet-derived growth factor, and so on), thereby stimulating residual normal myocytes for regeneration and proliferation and intrinsic myocardial stem cells (endogenous stem cells) for cell regeneration and for cell fusion (27–31).

Mitotic indexes are three to four times more frequent within the border zone of myocardial necrosis when compared with non-injured heart muscle (26). Moreover, 20% to 40% of intracoronarily transplanted bone-marrow-derived stem cells may be accumulated within the border zone of MI. There were no signs of apparent microcirculation disturbances because all patients had Thrombolysis In Myocardial Infarction flow grade 3. Thus, it is conceivable that in MI the border zone represents the optimum “niche” for exogenously transplanted stem cells, stimulating mitosis rates and heart muscle regeneration, preferably originating in and expanding from these areas. Cell fusion may also contribute to heart muscle regeneration, which takes its origin from the border zone, expanding gradually to the necrotic core of the infarcted area.

Our study cannot determine which cell-biologic and molecular mechanisms are responsible for heart muscle repair or which of the studied factors may play the predominant role. However, the final functional outcome of this cell therapy demonstrates three main target effects: improvement in muscle function (pumping ability and contractility), myocardial perfusion (SPECT), and myocardial glucose metabolism (PET), thus giving evidence that heart muscle repair must have taken place by this intracoronary bone marrow cell transplantation procedure.

The clinical significance of this novel therapeutic approach may embrace a large number of patients with chronic coronary artery disease, preferably after previous or long-standing MI. It is conceivable that remodeling after infarction may be ameliorated or even stopped by this procedure. Thus, cell therapy may represent a new option of basic and causal therapy in chronic infarcted myocardium. It is an open question whether variations of the amount and kind of bone marrow cells, the administration technique, and the transplantation procedure itself, by enhanced environment and improvement of the angiogenic micromilieu, can further improve the milieu-dependent differentiation or regeneration of bone marrow cells in chronic infarcted heart disease. Therefore, our clinical results represent a stable basis to proceed to the next necessary step: to a larger prospective randomized study.

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EVIDENCE APPENDIX

ITEM NO. 12

**Alberts et al. 2002 publication in Molecular Biology of the Cell,
4th Ed., Chapter 17, cited by the Examiner in
October 2, 2008 Office Action**


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Molecular Biology of the Cell

Bruce Alberts
Alexander Johnson
Julian Lewis
Martin Raff
Keith Roberts
Peter Walter

Molecular Biology of the Cell is the classic in-depth text reference in cell biology. By extracting fundamental concepts and meaning from this enormous and ever-growing field, the authors tell the story of cell biology, and create a coherent framework through which non-expert readers may approach the subject. Written in clear and concise language, and illustrated with original drawings, the book is enjoyable to read, and provides a sense of the excitement of modern biology. Molecular Biology of the Cell not only sets forth the current understanding of cell biology (updated as of Fall 2001), but also explores the intriguing implications and possibilities of that which remains unknown.

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Biochemistry and Biophysics at the University of California, San Francisco. **Alexander Johnson** received his Ph.D. from Harvard University and is a Professor of Microbiology and Immunology at the University of California, San Francisco. **Julian Lewis** received his D.Phil. from the University of Oxford and is a Principal Scientist at the Imperial Cancer Research Fund, London. **Martin Raff** received his M.D. from McGill University and is at the Medical Research Council Laboratory for Molecular Cell Biology and Cell Biology Unit and in the Biology Department at University College London. **Keith Roberts** received his Ph.D. from the University of Cambridge and is Associate Research Director at the John Innes Centre, Norwich. **Peter Walter** received his Ph.D. from The Rockefeller University in New York and is Professor and Chairman of the Department of Biochemistry and Biophysics at the University of California, San Francisco, and an Investigator of the Howard Hughes Medical Institute.

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Cell Biology Interactive

Artistic and Scientific Direction: Peter Walter

Narrated by: Julie Theriot

Production, Design, and Development: Mike Morales

Front cover Human Genome: Reprinted by permission from Nature, International Human Genome Sequencing Consortium, 409:860-921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and

influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila*, *Arabidopsis*, Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

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EVIDENCE APPENDIX

ITEM NO. 13

**4th Supplemental Declaration of Dr. Heuser cited by Appellant
as Exhibit A in the Amendment dated June 26, 2006**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: Elizabeth C. Kemmerer
)	
FILED: April 17, 2001)	
)	GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING)	
MUSCLE IN A HUMAN HEART)	

**FOURTH SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.**

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. This Fourth Supplemental Declaration is submitted in addition to my previous Declaration, dated June 5, 2003, my Supplemental Declaration dated February 4, 2004, my Second Supplemental Declaration dated July 18, 2004, and my Third Supplemental Declaration dated February 15, 2005. No changes are made to any of such previous Declarations.
3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my Declaration of June 5, 2003, and my background is further amplified by materials submitted in my Second and Third Supplemental Declarations.

4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood in my previous Declaration and Supplemental Declaration. A copy of such disclosures is attached hereto as Fourth Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 37, lines 19-25; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Fourth Supplemental Declaration Exhibit B.

5. The disclosures in Fourth Supplemental Declaration Exhibit A, also contained in my previous Declaration and Supplemental Declaration, relate to using growth factors, including cells, for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle. Such disclosures are also directed to the growth of new arteries in the heart.

I understand that the additional disclosures in Fourth Supplemental Declaration Exhibit B relate to using cellular growth factors, including bone marrow stem cells, to grow soft tissue, including an artery. Stem cells harvested from bone marrow, peripheral blood and from culture banks are described as being implanted for promoting morphogenesis and growth of all three-germ tissue layers, i.e. mesoderm, ectoderm and endoderm tissues. It would be understood by one skilled in the art that morphogenesis includes the growth of an artery, which comprises mesodermal tissue.

6. I have read and understood the claims set forth in Fourth Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in this application with this Fourth Supplemental Declaration.
7. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in the disclosures referenced therein, would be enabled to practice the method set forth in Fourth Supplemental Declaration Exhibit C and to predictably anticipate the results defined therein without need for resorting to undue experimentation. It is my further opinion that one skilled in the art reading such disclosures would understand that all of the well known administration procedures described at page 45 of the patent application, including intravenous, intraluminal, intramuscular, and with an angioplasty balloon, would be applicable for use in growing an artery in a human patient regardless of whether the genetic material was a gene; cell, including stem cells such as bone marrow stem cells; or another type of growth factor.

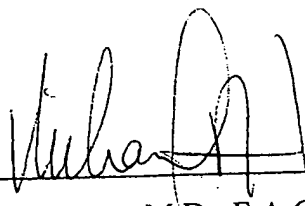
Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

6/16/00



Richard Heuser, M.D., F.A.C.C., F.A.C.P.

**FOURTH
SUPPLEMENTAL
DECLARATION**

EXHIBIT A

DISCLOSURES

EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

**FOURTH
SUPPLEMENTAL
DECLARATION**

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
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PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12– 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13– 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

PAGE 53, LINE 1 – PAGE 56, LINE 25

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a

single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting

a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth

factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 62, LINES 1-10

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

**FOURTH
SUPPLEMENTAL
DECLARATION**

EXHIBIT C

CLAIMS

EXHIBIT C
CLAIMS
APPLICATION SERIAL NO. 09/836,750

- Claim 236 A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.
- Claim 238 The method of claim 236, further comprising repairing a dead portion of said heart.
- Claim 239 The method of claim 236, further comprising repairing a damaged portion of said heart.
- Claim 243 The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
- Claim 244 The method of claim 243, wherein said growth factor comprises a cell.
- Claim 245 The method of claim 244, wherein said cell is multifactorial and non-specific.
- Claim 246 The method of claim 245, wherein said cell comprises a stem cell.
- Claim 247 The method of claim 236, wherein said growth factor is placed in said patient by injection.
- Claim 248 The method of claim 247, wherein said injection is intravenous.
- Claim 249 The method of claim 247, wherein said injection is intraluminal.
- Claim 250 The method of claim 247, wherein said injection is intramuscular.

- Claim 251 The method of claim 236, wherein said growth factor is placed in said patient by a carrier.
- Claim 252 The method of claim 251, wherein said carrier comprises an angioplasty balloon.
- Claim 253 The method of claim 236, wherein said growth factor comprises a gene and a cell.
- Claim 257 The method of claim 236, wherein said growth factor is locally placed in said body.
- Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.
- Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.
- Claim 260 The method of claim 243, wherein said growth factor is locally placed in said body.
- Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 264 A method of growing a new portion of a pre-existing heart comprising locally placing a growth factor comprising a stem cell in a body of a human patient to grow new cardiac muscle in said heart.

- Claim 265 The method of claim 264, wherein said stem cell is placed in said patient by injection.
- Claim 266 The method of claim 264, wherein said stem cell comprises living stem cells harvested from bone marrow.
- Claim 267 The method of claim 266, wherein said stem cell is placed in said patient by injection.
- Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.
- Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.
- Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.
- Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.
- Claim 272 The method of claim 265, wherein said stem cell is injected into said heart.
- Claim 273 The method of claim 267, wherein said stem cell is injected into said heart.
- Claim 274 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.
- Claim 275 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.

- Claim 276 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.
- Claim 277 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.
- Claim 278 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.
- Claim 279 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.
- Claim 280 The method of claim 236 further comprising determining blood flow through said newly grown artery.
- Claim 281 The method of claim 238 further comprising determining blood flow through said newly grown artery.
- Claim 282 The method of claim 239 further comprising determining blood flow through said newly grown artery.
- Claim 283 The method of claim 236 further comprising observing said newly grown artery.
- Claim 284 The method of claim 238 further comprising observing said newly grown artery.
- Claim 285 The method of claim 239 further comprising observing said newly grown artery.

- Claim 286 A method of repairing a dead portion of a pre-existing heart comprising the steps of placing stem cells adjacent said dead portion; forming a new artery in said heart, thereby causing said dead portion of said heart to be repaired.
- Claim 287 The method of claim 286, wherein said stem cells are placed by injection.
- Claim 288 The method of claim 286, wherein said stem cells are placed by intraluminal administration.
- Claim 289 The method of claim 286, wherein said stem cells are placed by an angioplasty balloon.
- Claim 290 A method of repairing a damaged portion of a pre-existing heart comprising the steps of placing stem cells adjacent said damaged portion; forming a new artery in said heart, thereby causing said damaged portion of said heart to be repaired.
- Claim 291 The method of claim 290, wherein said stem cells are placed by injection.
- Claim 292 The method of claim 290, wherein said stem cells are placed by intraluminal administration.
- Claim 293 The method of claim 290, wherein said stem cells are placed by an angioplasty balloon.

EVIDENCE APPENDIX

ITEM NO. 14

**3rd Supplemental Declaration of Dr. Andrew E. Lorincz cited
by Appellant as Exhibit B in the Amendment
dated June 26, 2006**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: Elizabeth C. Kemmerer
)	
FILED: April 17, 2001)	
)	
FOR: METHOD FOR GROWING)	GROUP ART UNIT: 1646
MUSCLE IN A HUMAN HEART)	

**THIRD SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I, Andrew E. Lorincz, declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.
2. This Third Supplemental Declaration is submitted in addition to my previous Declaration, dated June 9, 2003, my Supplemental Declaration dated February 3, 2004, and my Second Supplemental Declaration dated July 19, 2004. No changes are made to any of such previous Declarations.
3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my Declaration of June 9, 2003, and my background is further amplified by materials submitted in my Second Supplemental Declaration.
4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood

in my previous Declaration and Supplemental Declaration. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit A.

I have also read and understood additional disclosures of the above-referenced patent application at page 33, lines 8-10; page 37, lines 19-25; page 40, line 20 through page 43 line 3; page 44, lines 12 and 13; page 48, lines 13-15; page 53, line 1 through page 56, line 25; and page 62, lines 1-10. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

5. The disclosures in Third Supplemental Declaration Exhibit A, also contained in my previous Declaration and Supplemental Declaration, relate to using growth factors, including cells, for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle. Such disclosures are also directed to the growth of new arteries in the heart.

I understand that the additional disclosures in Third Supplemental Declaration Exhibit B relate to using cellular growth factors, including bone marrow stem cells, to grow soft tissue, including an artery. Stem cells harvested from bone marrow, peripheral blood and from culture banks are described as being implanted for promoting morphogenesis and growth of all three-germ tissue layers, i.e. mesoderm, ectoderm and endoderm tissues. It would be understood by one skilled in the art that morphogenesis includes the growth of an artery, which comprises mesodermal tissue.

6. I have read and understood the claims set forth in Third Supplemental Declaration Exhibit C and have been informed that such claims will be concurrently presented in this application with this Third Supplemental Declaration.

7. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in the disclosures referenced therein, would be enabled to practice the method set forth in Third Supplemental Declaration Exhibit C and to predictably anticipate the results defined therein without need for resorting to undue experimentation. It is my further opinion that one skilled in the art reading such disclosures would understand that all of the well known administration procedures described at page 45 of the patent application, including intravenous, intraluminal, intramuscular, and with an angioplasty balloon, would be applicable for use in growing an artery in a human patient regardless of whether the genetic material was a gene; cell, including stem cells such as bone marrow stem cells; or another type of growth factor.

Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 5 June 2006

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

**THIRD
SUPPLEMENTAL
DECLARATION**

EXHIBIT A

DISCLOSURES

EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

**THIRD
SUPPLEMENTAL
DECLARATION**

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
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PAGE 33, LINES 8-10

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 37, LINES 19-25

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, clone cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 40, LINE 20 – PAGE 43, LINE 3

EXAMPLE 11

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MXS-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

MXS-1 and MXS-2 transcription factors are obtained which will initiate the expression of the MXS-1 and MXS-2 homeobox genes.

The MXS-1 and MXS-2 transcription factors, BMP-2 and BMP-4 bone morphogenic proteins, and MXS-1 and MXS-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 12

Example 11 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 13

Example 11 is repeated except that the MXS-1 and MXS-2 transcription factors are not utilized. The transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

Example 13 is repeated except that the stem cells are starved and the transcription of the MXS-1 and MXS-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 15

WT-1 and PAX genes are obtained from a sample of skin tissue is removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees.

The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factors and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The kidney germ is transplanted in the patient's body near the optic nerve. As the kidney grows, its blood supply will be derived from nearby arteries.

EXAMPLE 17

The Aniridia gene is obtained from a sample of skin tissue is removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 44, LINES 12- 13

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 48, LINES 13- 15

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur in vivo, ex vivo, or in vitro.

PAGE 53, LINE 1 - PAGE 56, LINE 25

EXAMPLE 18

A 36 year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F to produce an genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a

single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site a new artery is growing adjacent the patient's original leg artery, and (2) at the second site a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting

a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient cultures, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials inserted ex vivo into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly timed transplantation, organ growth completes itself.

During the ex vivo application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In in vivo or ex vivo embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 19

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace in vivo a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injected intramuscularly. Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736 bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth

factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open heart surgery, endoscopic surgery, direction injection of the needle without incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart. The other end of the artery branches into increasing smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both in vitro and in vivo. RAOTS external ultrasound gives a semiquantitative analysis of arterial flow. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 20

A patient, a forty year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five second increments and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 62, LINES 1-10

EXAMPLE 36

Example 18 is repeated except that the patient is a 55 year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

**THIRD
SUPPLEMENTAL
DECLARATION**

EXHIBIT C

CLAIMS

EXHIBIT C
CLAIMS
APPLICATION SERIAL NO. 09/836,750

- Claim 236 A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.
- Claim 238 The method of claim 236, further comprising repairing a dead portion of said heart.
- Claim 239 The method of claim 236, further comprising repairing a damaged portion of said heart.
- Claim 243 The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
- Claim 244 The method of claim 243, wherein said growth factor comprises a cell.
- Claim 245 The method of claim 244, wherein said cell is multifactorial and non-specific.
- Claim 246 The method of claim 245, wherein said cell comprises a stem cell.
- Claim 247 The method of claim 236, wherein said growth factor is placed in said patient by injection.
- Claim 248 The method of claim 247, wherein said injection is intravenous.
- Claim 249 The method of claim 247, wherein said injection is intraluminal.
- Claim 250 The method of claim 247, wherein said injection is intramuscular.

- Claim 251 The method of claim 236, wherein said growth factor is placed in said patient by a carrier.
- Claim 252 The method of claim 251, wherein said carrier comprises an angioplasty balloon.
- Claim 253 The method of claim 236, wherein said growth factor comprises a gene and a cell.
- Claim 257 The method of claim 236, wherein said growth factor is locally placed in said body.
- Claim 258 The method of claim 238, wherein said growth factor is locally placed in said body.
- Claim 259 The method of claim 239, wherein said growth factor is locally placed in said body.
- Claim 260 The method of claim 243, wherein said growth factor is locally placed in said body.
- Claim 261 The method of claim 236, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 262 The method of claim 238, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 263 The method of claim 239, wherein said growth factor comprises living stem cells harvested from bone marrow.
- Claim 264 A method of growing a new portion of a pre-existing heart comprising locally placing a growth factor comprising a stem cell in a body of a human patient to grow new cardiac muscle in said heart.

- Claim 265 The method of claim 264, wherein said stem cell is placed in said patient by injection.
- Claim 266 The method of claim 264, wherein said stem cell comprises living stem cells harvested from bone marrow.
- Claim 267 The method of claim 266, wherein said stem cell is placed in said patient by injection.
- Claim 268 The method of claim 262, wherein said stem cell is placed in said patient by injection.
- Claim 269 The method of claim 263, wherein said stem cell is placed in said patient by injection.
- Claim 270 The method of claim 258, wherein said growth factor comprises a cell and said cell is placed adjacent to said dead portion of said heart.
- Claim 271 The method of claim 259, wherein said growth factor comprises a cell and said cell is placed adjacent to said damaged portion of said heart.
- Claim 272 The method of claim 265, wherein said stem cell is injected into said heart.
- Claim 273 The method of claim 267, wherein said stem cell is injected into said heart.
- Claim 274 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.
- Claim 275 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intravenous injection.

- Claim 276 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.
- Claim 277 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by intraluminal injection.
- Claim 278 The method of claim 238, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.
- Claim 279 The method of claim 239, wherein said growth factor comprises a cell and said cell is placed in said body by an angioplasty balloon.
- Claim 280 The method of claim 236 further comprising determining blood flow through said newly grown artery.
- Claim 281 The method of claim 238 further comprising determining blood flow through said newly grown artery.
- Claim 282 The method of claim 239 further comprising determining blood flow through said newly grown artery.
- Claim 283 The method of claim 236 further comprising observing said newly grown artery.
- Claim 284 The method of claim 238 further comprising observing said newly grown artery.
- Claim 285 The method of claim 239 further comprising observing said newly grown artery.

- Claim 286 A method of repairing a dead portion of a pre-existing heart comprising the steps of placing stem cells adjacent said dead portion; forming a new artery in said heart, thereby causing said dead portion of said heart to be repaired.
- Claim 287 The method of claim 286, wherein said stem cells are placed by injection.
- Claim 288 The method of claim 286, wherein said stem cells are placed by intraluminal administration.
- Claim 289 The method of claim 286, wherein said stem cells are placed by an angioplasty balloon.
- Claim 290 A method of repairing a damaged portion of a pre-existing heart comprising the steps of placing stem cells adjacent said damaged portion; forming a new artery in said heart, thereby causing said damaged portion of said heart to be repaired.
- Claim 291 The method of claim 290, wherein said stem cells are placed by injection.
- Claim 292 The method of claim 290, wherein said stem cells are placed by intraluminal administration.
- Claim 293 The method of claim 290, wherein said stem cells are placed by an angioplasty balloon.

EVIDENCE APPENDIX

ITEM NO. 15

**Third Supplemental Declaration
Of Richard Heuser
(filed in co-pending application SN10/179,589)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 10/179,589)	
)	Examiner: Daniel C. Gamett
Filed: June 25, 2002)	
)	
For: METHOD FOR GROWING)	
HUMAN ORGANS AND)	
SUBORGANS)	

**THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.**

I Richard Heuser declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. My Curriculum Vitae was attached as Exhibit A to my Declaration of November 16, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of February 15, 2005 provide additional information regarding my background and experience.
3. I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Third Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4. I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Third Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20 line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Third Supplemental Declaration Exhibit C.

5. I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Third Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Third Supplemental Declaration Exhibit C.
6. In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Third Supplemental Declaration Exhibit E. Note that in two of

the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Third Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

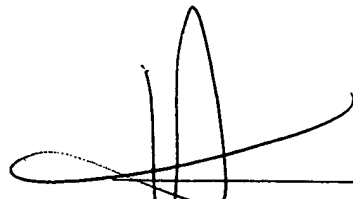
7. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

4/20/07



Richard Heuser

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT A

**MARCH 7, 2007
OFFICE ACTION
Paragraph 11, pages 7-9**

11. Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's) possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. According to the method for extrapolation provided in the footnote to pages 10-11, 250 μg of plasmid DNA (an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6 , and therefore the Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents is not included in the specification as filed. This is important because one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of μg of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage as purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed to the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

another. Therefore, contrary to Applicants assertion on page 9, the specification does not describe any dosage of cells to use to promote artery growth.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT C

**ADDITIONAL
DISCLOSURES**

EXHIBIT C
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1- 3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEGF or BMP growth factors produced by VEGF or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8-17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

**THIRD SUPPLEMENTAL
DECLARATION**

EXHIBIT D

CONVERSION

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6 , respectively.

THIRD SUPPLEMENTAL DECLARATION

EXHIBIT E

PUBLICATIONS (3)

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under

therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in $\mu\text{g.} \times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/ DNAP
Mean	8.64	6.33	0.75
S.E. of obs.	2.89	3.03	0.326
Observed range	4.0-15.0	2.1-13.5	0.43-1.9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8.75	7.59	0.90
S.E. of obs.	3.05	3.72	0.30
Observed range	3.9-17.4	2.0-17.4	0.3-1.8

Table 2. Cases of pernicious anaemia and other megaloblastic anaemias

NAP in $\mu\text{g.} \times 10^{-7}$ per cell

Group as a whole		DNAP 28 obs. on 12 cases	RNAP	Ratio DNAP/RNAP 28 obs. on 13 cases
	Mean	12.6	10.9	0.87
	S.E.	4.56	5.03	0.27
	Observed range	6.6-22.8	2.3-25.1	0.35-1.5
Group prior to therapy	Mean	12 obs. on 12 cases 12.67	11 obs. on 11 cases 13.38	12 obs. on 12 cases 1.06
	S.E.	4.17	5.19	0.249
	Observed range	8.1-22.8	7.5-25.1	0.69-1.5
Group during the course of therapy	Mean	17 obs. on 8 cases 12.63	15 obs. on 8 cases 9.09	16 obs. on 9 cases 0.73
	S.E.	4.36	4.21	0.198
	Observed range	6.6-18.8	2.3-17.6	0.35-1.0

Table 3. t test of significance between means

	P	DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	Degrees of freedom	44	44	46
	P	<0.001	<0.001	0.2-0.1
Megaloblastic series before therapy compared with normal	Degrees of freedom	28	29	30
	P	0.01-0.001	<0.001	0.01-0.001
	Degrees of freedom	28	29	30
Megaloblastic series during therapy compared with normal	Degrees of freedom	33	33	34
	P	0.01-0.001	0.05-0.02	0.8-0.7
	Degrees of freedom	33	33	34
Megaloblastic series before and during therapy compared	Degrees of freedom	27	24	26
	P	0.7-0.6	0.05-0.02	<0.001
	Degrees of freedom	27	24	26

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. BUFFA, W. D. LOTSPEICH, R. A. PETERS and R. W. WAKELIN. (*Department of Biochemistry, University of Oxford*)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluoro-tricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalosuccinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the

Table 1. *Rat heart aconitase*

Time (min.)	Citric acid (μmol.)	
	0	60
Additions:		
<i>cis</i> -Aconitate (5 μmol.)	0.21	3.90
<i>cis</i> -Aconitate + 'active' fraction	0.08	3.96
Citrate (5 μmol.)	4.90	4.34
Citrate + 'active' fraction	5.27	4.38

Table 2. *Pig heart isocitric dehydrogenase*

	<i>E</i> _{440 mμ.} (max. value)
DL-isocitrate only	0.078-0.085
Same + 'active' fraction	0.075
Same + <i>p</i> -chloromercuribenzoic acid 1.33 × 10 ⁻⁴ M	0.004

Table 3. *Pig heart oxalosuccinic decarboxylase*
(CO₂ evolution from 10 μmol. oxalosuccinate in 30 min. at 13.5°C. Net values)

	CO ₂ (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

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- Adler, E., v. Euler, H., Günther, G. & Plass, M. (1939). *Biochem. J.* 33, 1028.
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Appendix

Nucleic Acids

Nucleic Acids

Content and Distribution

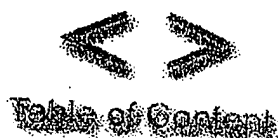
Nucleic acids in an average human cell

DNA	
Coding sequences	~6 pg/cella
Number of genes	3% of genomic DNA
Active genes	$0.51.0 \times 10^5$
	1.5×10^4
Total RNA	
rRNAs	~10 50 pg/cellb
tRNAs, snRNAs, and low mol. wt. RNA	80 85% of total RNA
mRNAs	15 20% of total RNA
nuclear RNA	1 5% of total RNA
Ratio of DNA:RNA in nucleus	~14% of total RNA
Number of mRNA moleculesc	~ 2:1
Number of different mRNA species	0.2 1.0×10^6
Low abundance mRNA (5 15 copies/cell)	$1.0 \ 3.4 \times 10^4$
Intermediate abundance mRNA (200 400 copies/cell)	11,000 different messages
High abundance mRNA (12,000 copies/cell)	500 different messages
Abundance of each message for:	<10 different messages
Low abundance mRNA (5 15 copies/cell)	<0.004% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	<0.1% of total mRNA
High abundance mRNA (12,000 copies/cell)	3% of total mRNA

- a 30 – 60 µg/ml blood for human leukocytes.
b 1 – 5 µg/ml blood for human leukocytes.
c Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/107 cells))	mRNA (µg/107 cells)
NIH/3T3 cells	75 200	
HeLa cells	100 300	1.5 4.0
CHO cells	200 400	2 6
		3 6



UMRECHNUNGSTABELLEN

I. Conversiontable

Molecular weight (daltons)	1µg	1nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA $\sim 3 \times 10^6$ nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (diploid)	0.03 pg
<i>E. coli</i>	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
<i>S. cerevisiae</i>	5 μm	66 μm^3
<i>S. pombe</i>	2 x 7 μm	22 μm^3
Mammalian cell	10-20 μm	500-4,000 μm^3
<i>E. coli</i>	1 x 3 μm	2 μm^3
Mammalian mitochondrion	1 μm	0.5 μm^3
Mammalian nucleus	5-10 μm	66-500 μm^3
Plant chloroplast	1 x 4 μm	3 μm^3
Bacteriophage lambda	50 nm (head only)	6.6 x 10 ⁻⁵ μm^3
Ribosome	30 nm diameter	1.4 x 10 ⁻⁵ μm^3
Globular monomeric protein	5 nm diameter	6.6 x 10 ⁻⁸ μm^3

III. Some useful concentrations

Total cell protein concentration Detergent soluble protein = 1-2 mg/ 10⁷ mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations

Nucleus (200 μm^3):

Abundant transcription factor

Rare transcription factor

1 nM (100,000 copies/ nucleus)

10 pM (1,000 copies/ nucleus)

Serum

50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	μg
10,000 Da protein	1

100,000 Da protein

10

Protein/ DNA conversions

1 kb of DNA encodes 333 amino acids $\approx 3.7 \times 10^4$ Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10 -30 pg
Proportion of total RNA in nucleus	$\sim 14\%$
DNA:RNA in nucleus	$\sim 2:1$
Human genome size (haploid)	3.3×10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	0.5 - 1×10^5
Active genes	1.5×10^4
mRNA molecules	2×10^5 - 1×10^6
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (μ g)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)			
	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)			
	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

	Leukocytes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O ₂ & CO ₂ transport
Cells per ml	4-7 x 10 ⁶	3-4 x 10 ⁸	5 x 10 ⁹
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNA content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2 x 10⁶ per ml in cases of immunosuppression, to 40 x 10⁶ during inflammation, to 500 x 10⁶ during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

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Mit Urteil vom 12. Mai 1998 hat das Landgericht Hamburg entschieden, dass man durch die Ausbringung eines Links die Inhalte der gelinkten Seite ggf. mit zu verantworten hat. Dies kann - so das LG - nur dadurch verhindert werden, dass man sich ausdrücklich von diesen Inhalten distanzier. Wir haben auf verschiedenen Seiten dieser Homepage Links zu anderen Seiten im Internet gelegt. Für all diese Links gilt: "Wir möchten ausdrücklich betonen, dass wir keinerlei Einfluss auf die Gestaltung und die Inhalte der gelinkten Seiten haben. Deshalb distanzieren wir uns hiermit ausdrücklich von allen Inhalten der gelinkten Seiten auf der Website inklusive aller Unterseiten und machen uns ihre Inhalte nicht zu eigen." Diese Erklärung gilt für alle auf der Homepage ausgebrachten Links und für alle Inhalte der Seiten, zu denen Links führen.

EVIDENCE APPENDIX

ITEM NO. 6

**Second Supplemental Declaration
Of Andrew E. Lorincz
(filed in co-pending application SN10/179,589)
(Exhibit D)**

EVIDENCE APPENDIX

ITEM NO. 16

**Second Supplemental Declaration
Of Andrew E. Lorincz
(filed in co-pending application SN10/179,589)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: James P. Elia)	
)	Group Art Unit: 1646
Serial No.: 10/179,589)	
)	Examiner: Elizabeth Kemmerer
Filed: June 25, 2002)	
)	
For: METHOD FOR GROWING)	
HUMAN ORGANS AND)	
SUBORGANS)	

**SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I, Andrew E. Lorincz, declare as follows:

1. I reside at 16135 NW 243rd Way, High Springs, Florida 32643-3813.
2. My Curriculum Vitae is attached was Exhibit A to my Declaration of November 8, 2004. Paragraph 3 of my Declaration and my Supplemental Declaration of June 5, 2006 provide additional information regarding my background and experience.
3. I have read the Examiner's criticism contained in paragraph 11, commencing on page 7 and ending on page 9 of the March 7, 2007 Office Action regarding the conversion of dosages of plasmid cDNA to dosages of cells. Such paragraph is set forth in Second Supplemental Exhibit A attached hereto. Specifically, I note the Examiner's criticism bridging pages 7 and 8 regarding the above-mentioned conversion that:

...one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular biology.

4. I have read and understood the disclosures of the above-referenced patent application at page 4, line 1 through page 5, line 14; at page 13, lines 3-10; at page 22, line 5 through page 24, line 15; and at page 26, line 3 through page 27, line 3. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit B.

I have also read and understood additional disclosures of the above-referenced patent application at page 9, lines 14-16; page 17, line 1 through page 20, line 8; page 21, lines 23 and 24; page 27, lines 1-3; page 28, lines 12-16; page 32, line 20 through page 39, line 19; and page 44, lines 8-17. A copy of such additional disclosures is attached hereto as Second Supplemental Declaration Exhibit C.

5. I have read and understood Applicant's conversion for dosages of plasmid cDNA to equivalent corresponding dosages of cells set forth in attached Second Supplemental Exhibit D as it relates to Examples 18 and 17 of the specification, which are contained in Second Supplemental Declaration Exhibit C.
6. In my opinion, the Examiner's criticism specifically delineated in Paragraph 3 above is not credible. Contrary to the Examiner's opinion, studies involving conversion of the average (mean) content of nucleic acids per cell in human marrow cells have been routinely conducted and accepted by skilled scientists for over 50 years. Three (3) publications illustrating the use of such well known conversion are included in the attached Second Supplemental Declaration Exhibit E. Note that in two of the publications, typical conversion results are set forth in tables, thereby eliminating the necessity to perform the actual calculation. Obviously, a sound scientific basis exists in the medical art for such conversions.

Further, those skilled in the art understand that DNA content is substantially consistent from tissues of any given species. Consequently, a skilled medical person relying on sound scientific bases at the time of the present invention would reasonably have understood how to extrapolate plasmid DNA to cells on a weight basis. Applicant's use of 40 pg as an average weight for nucleic acids in a human cell is fairly representative. Thus, I find Applicant's conversion set forth in the attached Second Supplemental Declaration Exhibit D to be consistent with the extrapolations set forth above and commonly used and relied upon by skilled persons in the medical art. Accordingly, the dosages specified in Examples 18 and 17 are sufficient to enable a person skilled in the medical art to convert dosages of plasmid DNA to corresponding dosages of genomic DNA within the context of Applicant's disclosed invention.

7. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 4-19-07

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

[illegible]

Figure 1. The structure of the proposed system.

[illegible][illegible]

11. Applicant admits on page 9 that Examples 17-19 employ nucleic acids, but asserts that one skilled in the art reading the specification, which teaches that cells, i.e., stem cells (BMC's) possess equivalent activity to genes (nucleic acids) and other genetic material in forming a new artery (i.e., promote morphogenesis of an organ—artery), would be able to easily extrapolate the number on a weight basis of mononuclear cells required to obtain equivalent results. According to the method for extrapolation provided in the footnote to pages 10-11, 250 μ g of plasmid DNA (an amount described in Examples 17 and 18) divided by 40 pg, (asserted to be is the average DNA content of a cell; the species of cell is not disclosed) equals 6.25×10^6 , and therefore the Examples 17 and 18 instruct the skilled artisan to use 6.25×10^6 cells. This argument is not persuasive for several reasons. First, this method of converting plasmid DNA to cell equivalents is not included in the specification as filed. This is important because one of skill in the art would never think to attempt such an extrapolation. The unsound scientific basis for the conversion of μ g of plasmid DNA to cellular equivalents would be obvious to anyone trained in molecular

biology. One basic assumption of the recited conversion is that the 40 pg of cellular DNA comprises the same gene dosage as a purified plasmid DNA. Every molecule of the postulated plasmid DNA comprises a copy of the VEGF cDNA. In contrast, VEGF coding sequences would comprise but one of 30-40 thousand genes in genomic DNA (at the time of filing, it was widely believed that the human genome comprised 100,000 genes). Therefore, one of skill in the art at the time of filing would not expect plasmid DNA and genomic DNA to be comparable on a per weight basis. Applicant's argument seems to view the living cell as little more than a container for DNA. The expression of the recombinant cDNA would be under control of the limited number of enhancer and promoter elements in the plasmid, as opposed to the native control elements with the genome. Therefore, even equivalent gene doses would not be expected to yield equivalent amounts of gene product with a plasmid as opposed to a cell. Applicant's argument seems to view the living cell as little more than a container for DNA. Delivery of the genes to a target as recombinant DNA as opposed to native genes within a living cell are technically different processes; there is no basis for using one to guide the other. For example, with DNA one is concerned with chemical stability, efficiency of uptake, stable retention, and subsequent expression of the injected molecule into target cells, whereas with cells separate issues of formation of effective attachment to ECM and neighboring cells, short- and long-term viability, and responses to environmental cues arise. As evidence, one need look no further than the US Patent classification system. Methods of *in vivo* treatments involving whole live cells as opposed to nucleic acids are separately classified: class 424 subclass 93.1 (cells); class 514, subclass 44 (polynucleotides). These separate classifications indicate a different status in the art such that it is well known that cell therapy and gene therapy are not obvious variants of one

Art Unit: 1647

another. Therefore, contrary to Applicants assertion on page 9, the specification does not describe any dosage of cells to use to promote artery growth.

SECOND SUPPLEMENTAL DECLARATION

EXHIBIT B

DISCLOSURES

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 4, LINE 1 – PAGE 5, LINE 14

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 13, LINES 3-10

Multifactorial and nonspecific cells (such as stem cells and germinal cells) can provide the necessary *in vivo* and *in vitro* cascade of genetic material once an implanted master control gene's transcription has been activated. Likewise, any host cell, cloned cell, cultured cell, or cell would work. Genetic switches (such as the insect hormone ecdysone) can be used to control genes inserted into humans and animals. These gene switches can also be used in cultured cells or other cells. Gene switches govern whether a gene is on or off making possible precise time of gene activity.

PAGE 22, LINE 5 – PAGE 24, LINE 15

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated

(taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth

factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

PAGE 26, LINE 3 – PAGE 27, LINE 3

Organs and/or tissues can be formed utilizing the patient's own cells. For example, a skin cell(s) is removed from the intraoral lining of a cheek. The cell is genetically screened to identify DNA damage or other structural and/or functional problems. Any existing prior art genetic screening technique can be utilized. Such methods can utilize lasers, DNA probes, PCR, or any other suitable device,. If the cell is damaged, a healthy undamaged cell is, if possible, identified and selected. If a healthy cell cannot be obtained, the damaged cell can be repaired by excision, alkylation, transition, or any other desired method. A growth factor(s) is added to the cell to facilitate dedifferentiation and then redifferentiation and morphogenesis into an organ or function specific tissue. Any machine known in the art can be used to check the genetic fitness of the organ and its stage of morphogenesis. A cell nutrient culture may or may not be utilized depending on the desired functional outcome (i.e., growth of an artery, of pancreatic Islet cells, of a heart, etc.) or other circumstances. Replantation can occur at any appropriate stage of

morphogenesis. The foregoing can be repeated without the patient's own cells if universal donor cells such as germinal cells are utilized. Germinal cells do not require a dedifferentiation. They simply differentiate into desired tissues or organs when properly stimulated. Similarly, the DNA utilized in the foreign procedure can come from the patient or from any desired source.

During reimplantation one of the patient's own cells is returned to the patient. During implantation, a cell not originally obtained from the patient is inserted on or in the patient.

In the example above, if germinal cells (and in some cases, stem cells) are utilized, a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

AND SUPPLEMENTAL
INFORMATION

EXHIBIT

ADDITIONAL
ENCLOSURE

EXHIBIT C
DISCLOSURES
APPLICATION SERIAL NO. 10/179,589

PAGE 9, LINES 14-16

Morphogenesis or morphogenetics is the origin and evolution of morphological characters and is the growth and differentiation of cells and tissues during development.

PAGE 17, LINE 1 – PAGE 20, LINE 8

EXAMPLE 10

MSX-1 and MSX-2 are the homeobox genes that control the generation and growth of a tooth. A sample of skin tissue is removed from the patient and the MSX-1 and MSX-2 homeobox gene(s) are removed from skin tissue cells. The genes are stored in an appropriate nutrient culture medium.

BMP-2 and BMP-4 growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 and 102 degrees F.

MSX-1 and MSX-2 transcription factors are obtained which will initiate the expression of the MSX-1 and MSX-2 homeobox genes.

The MSX-1 and MSX-2 transcription factors, BMP-2 and MBP-4 bone morphogenic proteins, and MSX-1 and MSX-2 genes are added to the nutrient culture medium along with the living stem cells.

EXAMPLE 11

Example 10 is repeated except that the transcription factors bind to a receptor complex in the stem cell nucleus.

EXAMPLE 12

Example 10 is repeated except that the MSX-1 and MSX-2 transcription factors are not utilized. The transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 13

Example 10 is repeated except that the stem cells are starved and the transcription of the MSX-1 and MSX-2 homeobox genes is activated by applying an electric spark to the nutrient culture medium.

EXAMPLE 14

WT-1 and PAX genes are obtained from a sample of skin tissue removed from the patient. The genes are stored in an appropriate nutrient culture medium. PAX genes produce PAX-2 and other transcription factors.

BMP-7 and other kidney related BMP growth factors are obtained by recombinant or natural extraction from bone.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The WT-1 and PAX genes, and BMP-7 and other kidney BMPS are added to the nutrient culture medium along with the living stem cells.

A primitive kidney germ is produced. The kidney germ is transplanted in the patient's body near a large artery. As the kidney grows, its blood supply will be derived from the artery.

EXAMPLE 15

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained.

Living stem cells are harvested from the bone marrow, the blood of the patient, or from cell culture techniques. The stem cells are placed in a nutrient culture medium at 98.6 degrees. The temperature of the culture medium can be varied as desired but ordinarily is between 40 to 102 degrees F.

The Aniridia transcription factor and growth factor and the Aniridia gene are added to the nutrient culture medium along with the living stem cells.

A primitive eye germ is produced. The eye germ is transplanted in the patient's body near the optic nerve. As the eye grows, its blood supply will be derived from nearby arteries.

EXAMPLE 16

The Aniridia gene is obtained from a sample of skin tissue removed from the patient. The gene(s) is stored in an appropriate nutrient culture medium.

Aniridia transcription factor (activates expression of the Aniridia gene) and growth factors (function to help stem cells differentiate during morphogenesis to form an eye) are obtained and added to the nutrient culture medium.

An eye germ develops. A branch of the nearby maxillary artery is translocated to a position adjacent the eye germ to promote the development of the eye germ. The eye germ matures into an eye which receives its blood supply from the maxillary artery.

The term "cell nutrient culture" as used herein can include any or any combination of the following: the extracellular matrix; conventional cell culture nutrients; and/or, a cell nutrient such as a vitamin. As such, the cell nutrient culture can be two-dimensional, three-dimensional, or simply a nutrient, and is useful in promoting the processes of cellular dedifferentiation, redifferentiation, differentiation, growth, and development.

PAGE 21, LINES 23– 24

An organ, as used herein, consists of two or more kinds of tissues joined into one structure that has a certain task.

PAGE 27, LINES 1- 3

In the example above, if germinal cells (and in some cases, stem cells) are utilized a direct differentiation and morphogenesis into an organ can occur *in vivo*, *ex vivo*, or *in vitro*.

PAGE 28, LINES 12-16

Avascular necrosis can be corrected with the insertion of a gene(s) and/or growth factor or other genetic material in the body. For example, avascular necrosis is diagnosed near a joint space. VEGF or BMP genes, or VEGF or BMP growth factors produced by VEGF or BMP genes, respectively, or any other desired genetic based material can be inserted to regrow blood vessels and/or bone.

PAGE 32, LINE 20 – PAGE 39, LINE 19

EXAMPLE 17

A 36-year old Caucasian male experiences pain in his left leg. A medical examination reveals a damaged one-inch long section of a large artery in his left leg. The examination also reveals that this damaged section of the artery is nearly completely clogged with plaque and that the wall of the artery is weakened. The weakening in the arterial wall makes attempting to clean out the artery risky and also makes it risky to attempt to insert a stent in the artery.

Recombinant cDNA encoded to combine with a cell ribosome to produce the human growth factor VEGF is assembled into a eukaryotic expression plasmid. The recombinant

cDNA is from cDNA libraries prepared from HL60 leukemia cells and is known to cause the growth of arteries. The plasmid is maintained at a room temperature of 76 degrees F.

The clones are placed in 1.0 milliliters of a normal saline carrier solution at a room temperature of 76 degrees F. to produce a genetic carrier solution. The genetic carrier solution contains about 250 ug of the cDNA clones. A nutrient culture can, if desired, be utilized in conjunction with or in place of the saline carrier. Each clone is identical. If desired, only a single clone can be inserted in the normal saline carrier solution. The saline carrier solution comprises 0.09% by weight sodium chloride in water. A saline carrier solution is selected because it will not harm the DNA clone.

Two sites are selected for injection of the genetic carrier solution. While the selection of sites can vary as desired, the sites are selected at the lower end (the end nearest the left foot of the patient) of the damaged section of the artery so that the new arterial section grown, can, if necessary, be used to take the place of the damaged section of the artery in the event the damaged section is removed.

The first site is on the exterior wall of the artery on one side of the lower end of the damaged section of the artery. A containment system is placed at the first site.

The second site is inside the wall of the artery on the other side of the lower end of the artery.

The genetic carrier solution is heated to a temperature of 98.6 degrees F. 0.25 milliliters of the genetic carrier solution is injected into the containment system at the first site. 0.25 milliliters of the genetic carrier solution is injected at the second site inside the wall of the artery. Care is taken to slowly inject the genetic carrier solution to avoid entry of the solution into the artery such that blood stream will carry away the cDNA in the solution.

After two weeks, an MRI is taken which shows the patient's leg artery. The MRI reveals new growth at the first and second sites.

After four weeks, another MRI is taken which shows the patient's leg artery. The MRI shows that (1) at the first site, a new artery is growing adjacent the patient's original leg artery, and (2) at the second site, a new section of artery is growing integral with the original artery, i.e., at the second site the new section of artery is lengthening the original artery, much like inserting a new section of hose in a garden hose concentric with the longitudinal axis of the garden hose lengthens the garden hose.

After about eight to twelve weeks, another MRI is taken which shows that the new artery growing adjacent the patient's original artery has grown to a length of about one inch and has integrated itself at each of its ends with the original artery such that blood flows through the new section of artery. The MRI also shows that the new artery at the second site has grown to a length of one-half inch.

In any of the examples of the practice of the invention included herein, cell nutrient culture can be included with the gene, the growth factor, the extracellular matrix, or the environmental factors.

In any of the examples of the practice of the invention included herein, the concept of gene redundancy can be applied. For example, the Examples 1 to 14 concerning a tooth list the genes MSX-1 and MSX-2. These genes differ by only two base pairs. Either gene alone may be sufficient. A further example of redundancy occurs in growth factors. Looking at the Examples 10 to 14, BMP4 or BMP2 alone may be sufficient. Redundancy can also be utilized in connection with transcription factors, extracellular matrices, environmental factors, cell nutrient culture, physiological nutrient cultures, vectors, promoters, etc.

One embodiment of the invention inserts genetic material (gene, growth factor, ECM, etc.) into the body to induce the formation of an organ. Similar inducing materials *ex vivo* into or onto a living cell in an appropriate physiological nurturing environment will also induce the growth of an organ. The VCSEL laser allows early detection in a living cell of a morphogenic change indicating that organ formation has been initiated. With properly time transplantation, organ growth completes itself.

During the *ex vivo* application of the invention, a gene and/or growth factor is inserted into a cell or a group of cells; an ECM or environmental factor(s) are placed around and in contact with a cell or group of cells; or, genetic material is inserted into a subunit of a cell to induce organ growth. An example of a subunit of a cell is an enucleated cell or a comparable artificially produced environment. In *in vivo* or *ex vivo* embodiments of the invention to induce the growth of an organ, the genes, growth factors, or other genetic material, as well as the environmental factors or cells utilized, can come from any desired source.

EXAMPLE 18

Genetically produced materials are inserted in the body to cause the body to grow, reproduce, and replace *in vivo* a clogged artery in the heart. This is an example of site-specific gene expression. A plasmid expression vector containing an enhancer/promoter is utilized to aid in the transfer of the gene into muscle cells. The enhancer is utilized to drive the specific expression of the transcriptional activator. After the enhancer drives the expression of the transcriptional activator, the transcriptional activator transactivates the muscle/artery genes. Saline is used as a carrier. Cardiac muscle can take up naked DNA injection intramuscularly.

Injecting plasmid DNA into cardiac (or skeletal) muscle results in expression of the transgene in cardiac myocytes for several weeks or longer.

Readily available off-the-shelf (RAOTS) cDNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL60 leukemia cells, are assembled in a RAOTS expression plasmid utilizing 736bp CMV promoter/enhancer to drive VEGF expression. Other RAOTS promoters can be utilized to drive VEGF expression for longer periods of time. Other RAOTS recombinant clones of angiogenic growth factors other than VEGF can be utilized, for example, fibroblast growth factor family, endothelial cell growth factor, etc. Downstream from the VEGF cDNA is an SV40 polyadenylation sequence. These fragments occur in the RAOTS pUC118 vector, which includes an Escherichia coli origin of replication and the Beta lactamase gene for ampicillin resistance.

The RAOTS construct is placed into a RAOTS 3 ml syringe with neutral pH physiologic saline at room temperature (or body temperature of about 37 degrees C). The syringe has a RAOTS 27 gauge needle.

Access to the cardiac muscle is gained by open-heart surgery, endoscopic surgery, direct injection of the needle with incision, or by any other desired means. The cardiac muscle immediately adjacent a clogged artery is slowly injected with the RAOTS construct during a five second time period. Injection is slow to avoid leakage through the external covering of muscle cells. About 0.5 ml to 1.0 ml (milliliter) of fluid is injected containing approximately 500 ug phVEGF165 in saline (N=18). The readily available off-the-shelf cDNA clones cause vascular growth which automatically integrates itself with the cardiac muscle. Anatomic evidence of collateral artery formation is observed by the 30th day following injection to the RAOTS construct. One end of the artery integrates itself in the heart wall to receive blood from the heart.

The other end of the artery branches into increasingly smaller blood vessels to distribute blood into the heart muscle. Once the growth of the new artery is completed, the new artery is left in place in the heart wall. Transplantation of the new artery is not required.

Blood flow through the new artery is calculated in a number of ways. For example, Doppler-derived flow can be determined by electromagnetic flowmeters (using, for example, a Doppler Flowmeter sold by Parks Medical Electronic of Aloha, Oregon) both *in vitro* and *in vivo*. Also, RAOTS angiograms or any other readily available commercial devices can be utilized.

VEGF gene expression can be evaluated by readily available off-the-shelf polymerase chain reaction (PCR) techniques.

If controls are desired, the plasmid pGSVLacZ containing a nuclear targeted Beta-galactosidase sequence coupled to the simian virus 40 early promoter can be used. To evaluate efficiency, a promoter-matched reporter plasmid, pCMV Beta (available from Clontech of Palo Alto, California), which encodes Beta-galactosidase under control of CMV promoter/enhancer, can be utilized. Other RAOTS products can be utilized if desired.

EXAMPLE 19

A patient, a forty-year old African-American female in good health, has been missing tooth number 24 for ten years. The space in her mouth in which her number 24 tooth originally resided is empty. All other teeth except tooth number 24 are present in the patient's mouth. The patient desires a new tooth in the empty "number 24" space in her mouth.

A full thickness mucoperiosteal flap surgery is utilized to expose the bone in the number 24 space. A slight tissue reflection into the number 23 tooth and number 25 tooth areas is carried out to insure adequate working conditions.

A Midwest Quietair handpiece (or other off-the-shelf handpiece) utilizing a #701 XXL bur (Dentsply Midwest of Des Plaines, Illinois) (a #700, #557, #558, etc. bur can be utilized if desired) is used to excavate an implant opening or site in the bone. The implant opening is placed midway between the roots of the number 23 and number 25 teeth. The opening ends at a depth which is about fifteen millimeters and which approximates the depth of the apices of the roots of the number 23 and number 25 teeth. Care is taken not to perforate either the buccal or lingual wall of the bone. In addition, care is taken not to perforate or invade the periodontal ligament space of teeth numbers 23 and 25.

An interrupted drilling technique is utilized to avoid overheating the bone when the #701XXL bur is utilized to form the implant opening. During a drilling sequence, the drill is operated in five-second increments; and the handpiece is permitted to stall. Light pressure and a gentle downward stroke are utilized.

PAGE 44, LINES 8-17

EXAMPLE 35

Example 17 is repeated except that the patient is a 55-year old Caucasian male, and the genetic carrier solution is injected into two sites in the coronary artery of the patient. The first site is on the exterior wall on one side of the artery. The second site is inside the wall of the artery on the other side of the artery. A section of the artery is damaged, is partially blocked, and has a weakened wall. The first and second sites are each below the damaged section of the

artery. Similar results are obtained, i.e., a new section of artery grows integral with the original artery, and a new section of artery grows adjacent the original artery. The new section of artery has integrated itself at either end with the original artery so that blood flows through the new section of artery.

AND SUPPLEMENT

TO

VOLUME II

CONVERSION

EXHIBIT D

CONVERSION

The conversion for dosages of nucleic acids to corresponding dosages of cells was conducted as follows. Examples 18 and 17 specified dosages of 500 micrograms (ug) and 250 ug, respectively. The weight of nucleic acids of an average cell was considered to equal 40 picograms (pg). The described dosages of 250 and 500 ug when converted to pg by multiplying by 10^6 equals 250×10^6 pg and 500×10^6 pg. Since nucleic acids of an average cell have an average weight of 40 pg, a conversion is made by dividing 250×10^6 and 500×10^6 by 40 to arrive at the equivalent cell dosages, which are 6.25×10^6 and 12.5×10^6 , respectively.

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Studies on the Average Content of Nucleic Acids in Human Marrow Cells. By J. N. DAVIDSON, I. LESLIE and J. C. WHITE. (From the Department of Biochemistry, University of Glasgow, and the Department of Pathology, Postgraduate Medical School of London)

In extension of previously reported analyses of the deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP) content of aspirated human bone marrow (Davidson, Leslie & White, 1947, 1948), we now report a modification involving enumeration of the nucleated cell content of the samples analysed. Results are expressed in terms of DNAP and RNAP per cell (Table 1), and are average values for the growing and adult cell populations of the analysed samples. The recent results of Vendrely & Vendrely (1948, 1949) and of Mirsky & Ris (1949) suggest a striking constancy in the DNAP content of normal cell nuclei from the tissues of any given species, and our figures for DNAP are of the same order as those quoted by the Vendrelys for human liver nuclei.

There is no significant difference between the means for the normal and the leukaemic series, either as a whole, or considering only acute leukaemia prior to therapy.

A small series of 6 cases of iron-deficiency anaemia has not shown significant variation of the mean DNAP and RNAP per cell from normal.

Results obtained from cases of pernicious and other megaloblastic anaemias are shown in Tables 2 and 3.

It must be noted clearly that the group under

therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy. The significant fall in RNAP from that in the group prior to therapy parallels the general increase in maturity of the marrow under therapy. Cases fully treated and returned to normal are under investigation.

Table 1

Normal human marrow

Values of Nucleic Acid Phosphorus (NAP) in $\mu\text{g.} \times 10^{-7}$ per cell

	DNAP 18 obs. on 16 individuals	RNAP 20 obs. on 18 individuals	Ratio RNAP/ DNAP
Mean	8.54	6.33	0.75
S.E. of obs.	2.89	3.03	0.326
Observed range	4.0-15.0	2.1-13.5	0.43-1.9

Marrow from cases of leukaemia of various types, before and during therapy

	28 obs. on 15 cases	24 obs. on 12 cases	
Mean	8.75	7.59	0.90
S.E. of obs.	3.05	3.72	0.30
Observed range	3.9-17.4	2.0-17.4	0.3-1.8

Table 2. Cases of pernicious anaemia and other megaloblastic anaemias

NAP in $\mu\text{g.} \times 10^{-7}$ per cell				
Group as a whole	Mean S.E. Observed range	DNAP 28 obs. on 12 cases 12.6 4.56 6.6-22.8	RNAP 10.9 5.03 2.3-25.1	Ratio DNAP/RNAP 28 obs. on 13 cases 0.87 0.27 0.35-1.5
		12 obs. on 12 cases 12.57 4.17 8.1-22.8	11 obs. on 11 cases 13.38 5.19 7.5-25.1	12 obs. on 12 cases 1.06 0.249 0.69-1.5
Group prior to therapy	Mean S.E. Observed range	17 obs. on 8 cases 12.63 4.36 6.6-18.8	15 obs. on 8 cases 9.09 4.21 2.3-17.6	16 obs. on 9 cases 0.73 0.198 0.35-1.0
Group during the course of therapy	Mean S.E. Observed range			

Table 3. t test of significance between means

	P	DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	Degrees of freedom	44 Highly significant	44 Highly significant	46 Not significant
Megaloblastic series before therapy compared with normal	Degrees of freedom	28 Highly significant	29 Highly significant	30 Highly significant
Megaloblastic series during therapy compared with normal	Degrees of freedom	33 Highly significant	33 Significant	34 Not significant
Megaloblastic series before and during therapy compared	Degrees of freedom	27 Not significant	24 Significant	26 Highly significant

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Fluoroacetate Poisoning and 'Jamming' of the Tricarboxylic Acid Cycle; Mode of Action of an 'Active' Fluoro Compound Synthesized via this Cycle. By P. BUFFA, W. D. LOTSPEICH, R. A. PETERS and R. W. WAKELIN. (Department of Biochemistry, University of Oxford)

So far no isolated enzyme has been inhibited by fluoroacetate. The hypothesis has been advanced by Liébecq & Peters (1949) (see also Martius, 1949) that the inhibition of citrate oxidation, occurring also *in vivo* (Buffa & Peters, 1949), is due to the 'jamming' effect of an enzymically synthesized fluorotricarboxylic acid in the Krebs tricarboxylic acid cycle. In support of this hypothesis, Buffa, Peters & Wakelin (1950) have isolated, from guinea-pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction, which is 'active' in preventing disappearance of added citrate. This active fraction is mainly citrate; it contains no fluoroacetate, but there is present a small amount of a F-compound which is chromatographically inseparable from the tricarboxylic acids.

We have tried to find the exact point of inhibition in the enzymes of the tricarboxylic acid cycle by determining the effect of the 'active' fractions upon aconitase (Johnson, 1939), isocitric dehydrogenase (Adler, Euler, Günther & Plass, 1939) and oxalosuccinic decarboxylase (Ochoa & Weiss-Tabori, 1948), obtained from rat and pig heart tissue. Tables 1, 2 and 3 show that the results were negative, even when amounts of 'active' fraction were used 80 times larger than those inhibiting citrate disappearance in the kidney homogenates.

All the evidence from experiments *in vivo* and *in vitro* (? mitochondrial homogenates) points to inhibition by the 'active' compound at either the

Table 1. Rat heart aconitase

Time (min.)	Citric acid (μmol.)	
	0	60
Additions:		
<i>cis</i> -Aconitate (5 μmol.)	0.21	3.90
<i>cis</i> -Aconitate + 'active' fraction	0.08	3.96
Citrate (5 μmol.)	4.90	4.34
Citrate + 'active' fraction	5.27	4.38

Table 2. Pig heart isocitric dehydrogenase

	<i>E</i> _{340 mμ.} (max. value)
DL-isocitrate only	0.076-0.085
Same + 'active' fraction	0.075
Same + <i>p</i> -chloromercuribenzoic acid 1.33 × 10 ⁻³ M	0.004

Table 3. Pig heart oxalosuccinic decarboxylase
(CO₂ evolution from 10 μmol. oxalosuccinate in 30 min. at 13.5°C. Net values)

	CO ₂ (μl.)
Enzyme alone	83
Enzyme + 'active' fraction	76
Enzyme + DL-isocitrate (control)	14

aconitase or isocitric dehydrogenase stage. Hence, we are led to the conclusion that the complete system has properties not present in its isolated enzyme components. Whether these be due to factors of organization or to missing components must be decided by further work.

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Appendix

Nucleic Acids

Nucleic Acids

Content and Distribution

Nucleic acids in an average human cell

DNA	
Coding sequences	~6 pg/cella
Number of genes	3% of genomic DNA
Active genes	$0.51.0 \times 10^5$
	1.5×10^4
Total RNA	
rRNAs	~10 50 pg/cellb
tRNAs, snRNAs, and low mol. wt. RNA	80 85% of total RNA
mRNAs	15 20% of total RNA
nuclear RNA	1 5% of total RNA
	~14% of total RNA
Ratio of DNA:RNA in nucleus	
Number of mRNA moleculesc	~ 2:1
Number of different mRNA species	0.2 1.0×10^6
Low abundance mRNA (5 15 copies/cell)	1.0 3.4×10^4
Intermediate abundance mRNA (200 400 copies/cell)	11,000 different messages
High abundance mRNA (12,000 copies/cell)	500 different messages
	<10 different messages
Abundance of each message for:	
Low abundance mRNA (5 15 copies/cell)	<0.004% of total mRNA
Intermediate abundance mRNA (200 400 copies/cell)	<0.1% of total mRNA
High abundance mRNA (12,000 copies/cell)	3% of total mRNA

- a 30 – 60 µg/ml blood for human leukocytes.
b 1 – 5 µg/ml blood for human leukocytes.
c Average size of mRNA molecule = 1930 bases.

RNA content of cells in culture

Type of cell	Total RNA (mRNA (µg/107 cells))	mRNA (µg/107 cells)
NIH/3T3 cells	75 200	1.5 4.0
HeLa cells	100 300	2 6
CHO cells	200 400	3 6

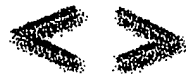


Table of Content

UMRECHNUNGSTABELLE.

I. Conversiontable

Molecular weight (daltons)	1µg	1nmole
100	10 nmoles or 6×10^{15} molecules	0.1 µg
1,000	1 nmole or 6×10^{14} molecules	1 µg
10,000	100 pmoles or 6×10^{13} molecules	10 µg
20,000	50 pmoles or 3×10^{13} molecules	20 µg
30,000	33 pmoles or 2×10^{13} molecules	30 µg
40,000	25 pmoles or 1.5×10^{13} molecules	40 µg
50,000	20 pmoles or 1.2×10^{13} molecules	50 µg
60,000	17 pmoles or 10^{13} molecules	60 µg
70,000	14 pmoles or 8.6×10^{12} molecules	70 µg
80,000	12 pmoles or 7.5×10^{12} molecules	80 µg
90,000	11 pmoles or 6.6×10^{12} molecules	90 µg
100,000	10 pmoles or 6×10^{12} molecules	100 µg
120,000	8.3 pmoles or 5×10^{12} molecules	120 µg
140,000	7.1 pmoles or 4.3×10^{12} molecules	140 µg
160,000	6.3 pmoles or 3.8×10^{12} molecules	160 µg
180,000	5.6 pmoles or 3.3×10^{12} molecules	180 µg
200,000	5 pmoles or 3×10^{12} molecules	200 µg

II. Some useful nucleotide dimensions

1 cm of DNA $\sim 3 \times 10^6$ nucleotides

Organism	Base pairs/ haploid genome	Base pairs/ diploid genome	Length/cell	Mass

Human	3×10^9	6×10^9	2 meters (diploid)	6 pg
Fly	1.65×10^8	3.3×10^8	100 cm (diploid)	0.3 pg
Yeast	1.35×10^7	2.7×10^7	10 cm (diploid)	0.03 pg
<i>E. coli</i>	4.7×10^6	-	1.5 cm (diploid)	0.0045 pg
SV40	5×10^3	-	1.7 nm	0.000006 pg

III. Some useful cell dimensions

Organism	Dimensions	Volume
<i>S. cerevisiae</i>	5 μm	66 μm^3
<i>S. pombe</i>	2 x 7 μm	22 μm^3
Mammalian cell	10-20 μm	500-4,000 μm^3
<i>E. coli</i>	1 x 3 μm	2 μm^3
Mammalian mitochondrion	1 μm	0.5 μm^3
Mammalian nucleus	5-10 μm	66-500 μm^3
Plant chloroplast	1 x 4 μm	3 μm^3
Bacteriophage lambda	50 nm (head only)	$6.6 \times 10^{-5} \mu\text{m}^3$
Ribosome	30 nm diameter	$1.4 \times 10^{-5} \mu\text{m}^3$
Globular monomeric protein	5 nm diameter	$6.6 \times 10^{-8} \mu\text{m}^3$

III. Some useful concentrations

Total cell protein concentration Detergent soluble protein = 1-2 mg/ 10^7 mammalian cells or 100-200 mg/ ml for soluble proteins only

Specific protein concentrations

Nucleus (200 μm^3):

Abundant transcription factor

1 nM (100,000 copies/ nucleus)
10 pM (1,000 copies/ nucleus)

Rare transcription factor

Serum

50-100 mg/ ml

IV. Some useful Conversiontables

Molar conversions for protein

100 pmol	μg
10,000 Da protein	1

100,000 Da protein

120

Protein/ DNA conversions

1 kb of DNA encodes 333 amino acids $\approx 3.7 \times 10^4$ Da

Protein	DNA
10,000 Da	270 dp
30,000 Da	810 dp
100,000 Da	2,7 dp

Nucleic acid content of a typical human cell

DNA per cell	~ 6 pg
Total RNA per cell	~ 10 -30 pg
Proportion of total RNA in nucleus	$\sim 14\%$
DNA:RNA in nucleus	$\sim 2:1$
Human genome size (haploid)	3.3×10^9 bp
Coding sequences/ genomic DNA	3%
Number of genes	0.5 - 1×10^5
Active genes	1.5×10^4
mRNA molecules	2×10^5 - 1×10^6
Typical mRNA size	1900 nt

RNA distribution in a typical mammalian cell

RNA species	Relative amount
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%

RNA content in various cells and tissues

Source		Total RNA	mRNA (μ g)
Cell cultures (10^7 cells)		30-500	0.3-25
	NIH/3T3	120	3
	HeLa	150	3
	COS-7	350	5
Mouse-developmental stages (per organism)			
	Unfertilized egg	0.43 ng	nd
	Oocyte	0.35 ng	nd

	2-cell	0.24 ng	nd
	8-16-cell	0.69 ng	nd
	32-cell	1.47 ng	nd
	13-day-old-embryo	450	13
Mouse tissue (100 mg)			
	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

nd = not determined

Human blood*: cell, DNA, RNA, and protein content

	Leukocytes	Thrombocytes	Erythrocytes
Function	Immune response	Wound closing	O ₂ & CO ₂ transport
Cells per ml	4-7 x 10 ⁶	3-4 x 10 ⁸	5 x 10 ⁹
DNA content	30-60 µg/ ml blood (6 pg/cell)		
RNA content	1-5 µg/ ml blood		
Hemoglobin content			~150 mg/ ml blood (30 pg/cell)
Plasma protein content		60-80 mg/ ml	

*From a healthy individual. The leukocyte concentration can vary from 2 x 10⁶ per ml in cases of immunosuppression, to 40 x 10⁶ during inflammation, to 500 x 10⁶ during leukemia. The DNA and RNA content will vary accordingly.

zum Hauptmenü

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EVIDENCE APPENDIX

ITEM NO. 17

Kornowski U.S. Patent No. 7,097,832



(12) **United States Patent**
Kornowski et al.

(10) Patent No.: **US 7,097,832 B1**
(45) Date of Patent: **Aug. 29, 2006**

(54) **INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW**

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(73) Assignee: **Myocardial Therapeutics, Inc.**, San Diego, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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§ 371 (c)(1),
(2), (4) Date: **Jun. 14, 2001**

(87) PCT Pub. No.: **WO00/57922**

PCT Pub. Date: **Oct. 5, 2000**

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(51) Int. Cl.
A01N 63/00 (2006.01)
C12N 5/00 (2006.01)
C12N 5/06 (2006.01)

(52) U.S. Cl. **424/93.7; 435/384; 435/372**

(58) Field of Classification Search **424/94.1, 424/577; 514/2, 21; 435/325**
See application file for complete search history.

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(57) **ABSTRACT**

A method of treating cardiac or myocardial conditions comprises the administration of an effective amount of autologous bone marrow. The bone marrow may optionally be stimulated and/or administered in combination with a pharmaceutical drug, protein, gene or other factor or therapy that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation or migration or blood vessel formation.

15 Claims, 2 Drawing Sheets

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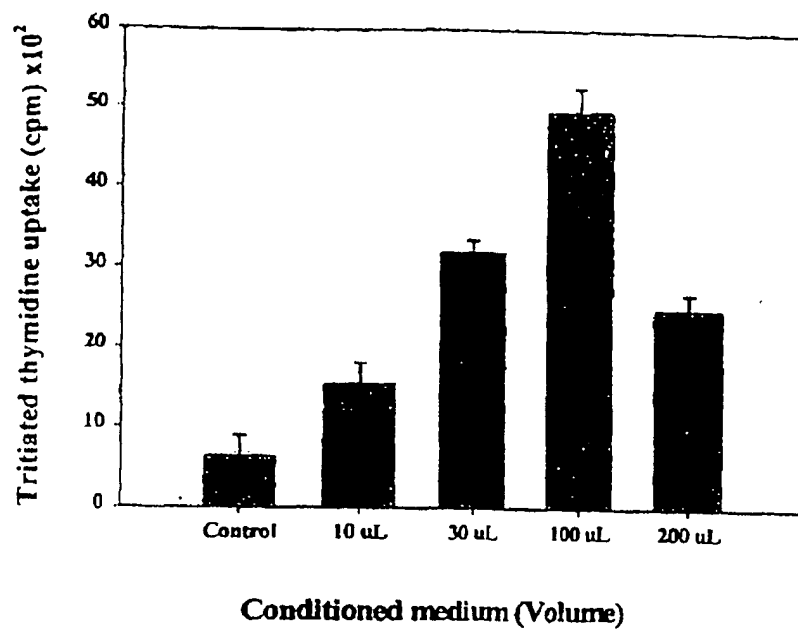


Fig. 1

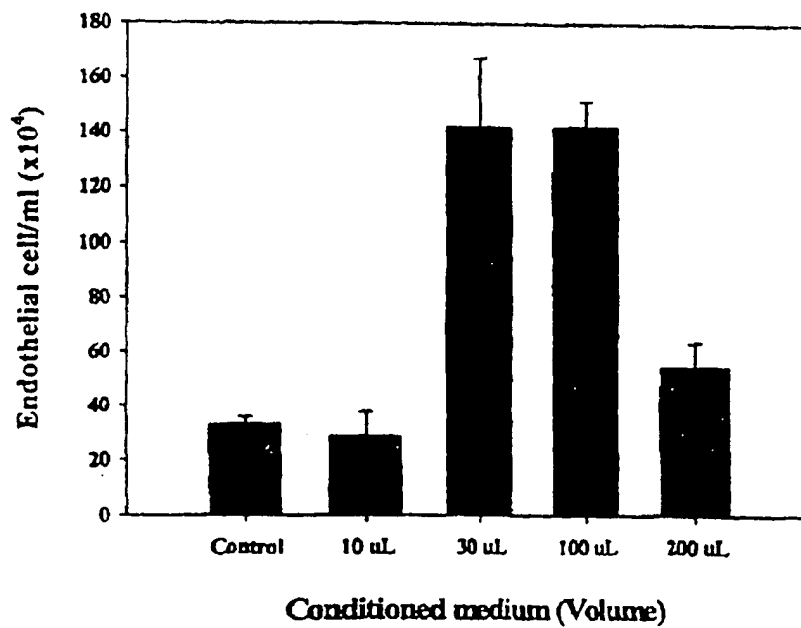


Fig. 2

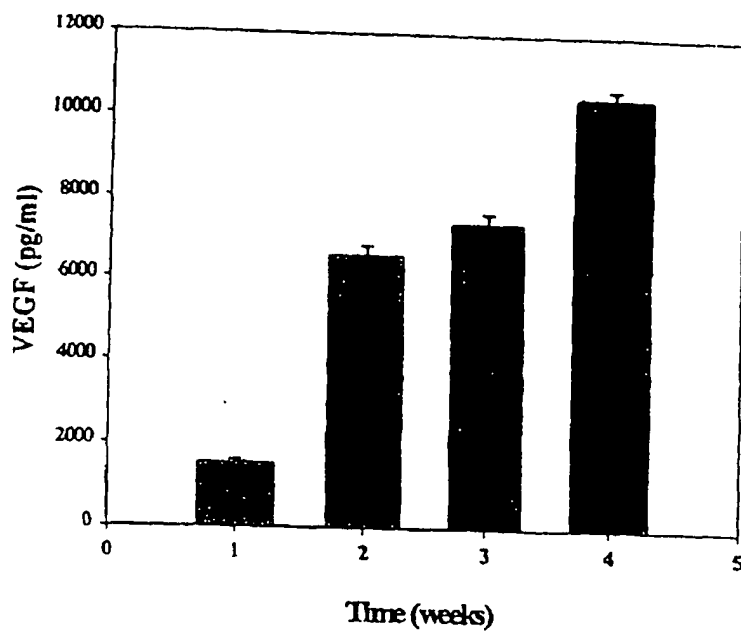


Fig. 3

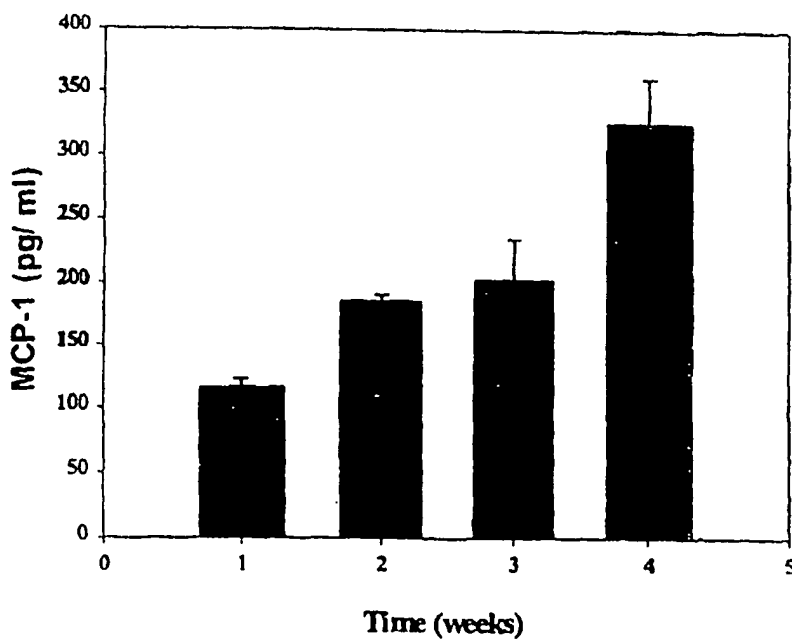


Fig. 4

INTRAMYOCARDIAL INJECTION OF AUTOLOGOUS BONE MARROW

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national stage application of international application PCT/US00/08353, filed 30 Mar. 2000, which claims benefit to U.S. Provisional Application Nos. 60/126,800, filed 30 Mar. 1999, and 60/138,379, filed 9 Jun. 1999.

FIELD OF THE INVENTION

This application is directed to a method of injecting autologous bone marrow. More specifically, this invention is directed to intramyocardial injection of autologous bone marrow to enhance collateral blood vessel formation and tissue perfusion.

BACKGROUND OF THE INVENTION

The use of recombinant genes or growth-factors to enhance myocardial collateral blood vessel function may represent a new approach to the treatment of cardiovascular disease. Komowski, R., et al., "Delivery strategies for therapeutic myocardial angiogenesis", *Circulation* 2000; 101:454-458. Proof of concept has been demonstrated in animal models of myocardial ischemia, and clinical trials are underway. Unger, E. F., et al., "Basic fibroblast growth factor enhances myocardial collateral flow in a canine model", *Am J Physiol* 1994; 266:H1588-1595; Banai, S. et al., "Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs", *Circulation* 1994; 83:2189; Lazarous, D. F., et al., "Effect of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart", *Circulation* 1995; 91:145-153; Lazarous, D. F., et al., "Comparative effects of basic development and the arterial response to injury", *Circulation* 1996; 94:1074-1082; Giordano, F. J., et al., "Intracoronary gene transfer of fibroblast growth factor-S increases blood flow and contractile function in an ischemic region of the heart", *Nature Med* 1996; 2:534-9. Most strategies for trans-catheter delivery of angiogenic factors have employed an intracoronary route which may have limitations due to imprecise localization of genes or proteins and systemic delivery to non-cardiac tissue. Thus, it would be desirable to have the capacity for direct delivery of angiogenic factors or genes to precisely defined regions of the myocardium rather than to the entire heart, and to minimize the potential for systemic exposure. Guzman, R. J., et al., "Efficient gene transfer into myocardium by direct injection of adenovirus vectors", *Circ Res* 1993; 73:1202-7; Mack, C. A., et al., "Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for VEGF-121, improves myocardial perfusion and function in the ischemic porcine heart", *J Thorac Cardiovasc Surg* 1998; 115:168-77.

The effect of direct intra-operative intramyocardial injection of angiogenic factors on collateral function has been studied in animal models of myocardial ischemia. Open chest, transepical administration of an adenoviral vector containing a transgene encoding an angiogenic peptide resulted in enhanced collateral function. (Mack et al., supra.) Angiogenesis was also reported to occur with direct intramyocardial injection of an angiogenic peptide or a

plasmid vector during open heart surgery in patients. Schumacher, B., et al., "Induction of neoangiogenesis in ischemic myocardium by human growth factors. First clinical results of a new treatment of coronary heart disease", *Circulation* 1998; 97:645-650; Losordo, D. W., et al., "Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia", *Circulation* 1998; 93:2800.

Despite the promising hope for therapeutic angiogenesis as a new modality to treat patients with coronary artery disease, there is still a huge gap regarding what specific strategy will optimally promote a clinically relevant therapeutic angiogenic response. Moreover, it is unclear which one (or more) out of multiple angiogenic growth factors may be associated with a beneficial angiogenic response. In addition, the use of different tissue delivery platforms, e.g., proteins, adenovirus, or "naked" DNA, to promote the optimal angiogenic response has remained an open issue.

OBJECTS OF THE INVENTION

It is an object of this invention to provide a novel therapeutic modality wherein autologous bone marrow is injected to promote angiogenesis in the injected tissue.

It is also an object of this invention to provide a novel method of intramyocardial injection to enhance collateral blood vessel formation and tissue perfusion.

These and other objects of the invention will become more apparent in the discussion below.

SUMMARY OF THE INVENTION

Most currently tested therapeutic approaches have focused on a single angiogenic growth factor (e.g., VEGF, FGF, angiopoietin-1) delivered to the ischemic tissue. This can be accomplished either by delivery of the end-product (e.g., protein) or by gene transfer, using diverse vectors. However, it is believed that complex interactions among several growth factor systems are probably necessary for the initiation and maintenance of new blood vessel formation. More specifically, it is believed important to induce a specific localized angiogenic milieu with various angiogenic cytokines interacting in concert and in a time-appropriate manner to initiate and maintain the formation and function of new blood vessels.

The bone marrow (BM) is a natural source of a broad spectrum of cytokines and cells that are involved in the control of angiogenic processes. It is therefore believed that the intramyocardial injection of autologous (A) BM, by taking advantage of the natural ability of these cells to secrete many angiogenic factors in a time-appropriate manner, provides an optimal intervention for achieving therapeutic collateral development in ischemic myocardium.

According to the invention autologous bone marrow is injected, either as a "stand alone" therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation. The "combined" agent(s) can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Non-limiting examples of these "combined" agents are Granulocyte-Monocyte Colony Stimulatory Factor (GM-CSF), Monocyte Chemoattractant Protein 1 (MCP-1), and Hypoxia Inducible Factor-1 (HIF-1). An example of an intervention that may enhance bone

marrow production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. The autologous bone marrow, alone or with "combined" agents, can be delivered to the patient directly via either trans-endocardial or trans-epicardial approaches into either ischemic and/or non-ischemic myocardium, or directly into any other ischemic organ (including a peripheral limb) to enhance and/or promote the development of collateral blood vessel formation and therefore collateral flow to ischemic myocardium or ischemic limbs. This approach can also be used to promote the development of newly implanted dedifferentiated and/or differentiated myocardial cells by the process of cardiac myogenesis.

The invention comprises various autologous bone marrow transplantation strategies to enhance angiogenesis and/or myogenesis and thereby accelerate the development of new blood vessels into ischemic myocardium or lower extremities. Another aspect of the invention concerns the strategy of "optimization of angiogenic gene expression." This strategy employs co-administration of HIF-1 with the autologous bone marrow. HIF-1 is a transcription factor known to be induced and activated by hypoxia, and known to induce expression of multiple genes involved in the response to hypoxia. A similar approach involves the exposure of autologous bone marrow to endothelial PAS domain protein 1 (EPAS1). EPAS1 shares high structural and functional homology with HIF-1. The strategy also involves the ex-vivo exposure of the bone marrow to hypoxia to increase the production of vascular endothelial growth factor (VEGF) expression or other cytokines with proven angiogenic activity (such as MCP-1) prior to its direct injection into the heart or any peripheral ischemic tissue. This invention thus includes the direct intramyocardial (trans-epicardial or trans-endocardial) or peripheral intramuscular injection of autologous bone marrow; stimulated autologous bone marrow, for example, stimulated by HIF-1, EPAS1, MCP-1, G μ M-CSF, or transient exposure to hypoxia or other forms of energy, such as ultrasound, RF, electromagnetic or laser energy; or autologous bone marrow product derived from conditioned medium (acellular component/s of cultured bone marrow). The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph of the proliferation of PAEC's vs. the quantities of conditioned medium;

FIG. 2 is a graph of the proliferation of endothelial cells vs. the quantities of conditioned medium;

FIG. 3 is a graph of the concentration of VEGF in conditioned medium over a four-week period of time; and

FIG. 4 is a graph of the concentration of MCP-1 in conditioned medium over a four-week period of time.

DETAILED DESCRIPTION OF THE INVENTION

Bone marrow is a natural source of a broad spectrum of cytokines that are involved in the control of angiogenic and inflammatory processes. The cytokines expressed comprise mediators known to be involved in the maintenance of early and late hematopoiesis (IL-1 alpha and IL-1 beta, IL-6, IL-7,

IL-8, IL-11 and IL-13; colony-stimulating factors, thrombopoietin, erythropoietin, stem cell factor, flt 3-ligand, hepatocyte cell growth factor, tumor necrosis factor alpha, leukemia inhibitory factor, transforming growth factors beta 1 and beta 3; and macrophage inflammatory protein 1 alpha). angiogenic factors (fibroblast growth factors 1 and 2, vascular endothelial growth factor) and mediators whose usual target (and source) is the connective tissue-forming cells (platelet-derived growth factor A, epidermal growth factor, transforming growth factors alpha and beta 2, oncostatin M and insulin-like growth factor-1), or neuronal cells (nerve growth factor). Sensebe, L., et al., *Stem Cells* 1997; 15:133-43. Moreover, it has been shown that VEGF polypeptides are present in platelets and megacaryocytes, and are released from activated platelets together with the release of beta-thromboglobulin. Wartiovaara, U., et al., *Thromb Haemost* 1998; 80:171-5; Mohle, R., *Proc Natl Acad Sci USA* 1997; 94:663-8.

There are also indicators to support the concept that angiogenesis is needed to support bone marrow function and development of hematopoietic cells, including stem cells and progenitor cells, that may enter the circulation and target to sites of wound healing and/or ischemia, ultimately contributing to new blood vessel formation. Monoclonal antibodies that specifically recognize undifferentiated mesenchymal progenitor cells isolated from adult human bone marrow have been shown to recognize cell surface markers of developing microvasculature, and evidence suggests such cells may play a role in embryonal angiogenesis. Fleming, J. E., Jr., *Dev Dyn* 1998; 212:119-32.

Bone marrow angiogenesis may become exaggerated in pathologic states where the bone marrow is being activated by malignant cells (such as in multiple myeloma) where bone marrow angiogenesis has been shown to increase simultaneously with progression of human multiple myeloma cells. Ribatti, D., et al., *Br J Cancer* 1999; 79:451-5. Moreover, vascular endothelial growth factor (VEGF) has been shown to play a role in the growth of hematopoietic neoplasms such as multiple myeloma, through either a paracrine or an autocrine mechanism. Bellamy, W. T., *Cancer Res* 1999; 59:728-33; Fiedler, W., *Blood* 1997; 89:1870-5). It is believed that autologous bone marrow, with its unique native humoral and cellular properties, is a potential source of various angiogenic compounds. This natural source of "mixed" angiogenic cytokines can surprisingly be utilized as a mixture of potent interactive growth factors to produce therapeutic angiogenesis and/or myogenesis; use of the cells per se could provide a more sustained source of these natural angiogenic agents.

One of the factors that most likely participates in initiating angiogenesis in response to ischemia is HIF-1, a potent transcription factor that binds to and stimulates the promoter of several genes involved in responses to hypoxia. Induction and activation of HIF-1 is tightly controlled by tissue pO₂: HIF-1 expression increases exponentially as pO₂ decreases, thereby providing a positive feedback loop by which a decrease in pO₂ causes an increase in the expression of gene products that serve as an adaptive response to a low oxygen environment. Activation of HIF-1 leads, for example, to the induction of erythropoietin, genes involved in glycolysis, and to the expression of VEGF. It probably also modulates the expression of many other genes that participate in the adaptive response to low pO₂ levels. The mechanism by which HIF-1 regulates levels of proteins involved in the response to hypoxia is through transcriptional regulation of genes responding to low pO₂. Thus, such genes have short DNA sequences within the promoter or enhancer regions

that contain HIF-1 binding sites, designated as hypoxia responsive elements (HRE). HIF-1 is a heterodimer with a basic helix-loop-helix motif, consisting of the subunits HIF-1 α and HIF-1 β . Its levels are regulated by pO₂ both transcriptionally and posttranscriptionally—HIF-1 induction is increased by hypoxia, and its half-life is markedly reduced as pO₂ levels increase.

It is relevant that while expression of HIF-1 (as determined in HeLa cells) is exponentially and inversely related to pO₂, the inflection point of the curve occurs at an oxygen saturation of 5%, with maximal activity at 0.5% and 1/2 maximal activity at 1.5–2.0%. These are relatively low levels of hypoxia, and it is not clear whether such levels occur in the presence of mild levels of myocardial or lower limb ischemia—i.e., levels present in the absence of tissue necrosis (myocardial infarction, and leg ulcerations, respectively). Thus, bone marrow cells could have the capacity to secrete angiogenic factors and thereby enhance collateral development. However, it is possible that such activity may not become manifest in the specific tissue environments treated unless some additional stimulus is present. It is, therefore, a preferred aspect of the invention to co-administer, if necessary, bone marrow implant with HIF-1. It is anticipated that HIF-1 will provide optimal expression of many of the hypoxia-inducible angiogenic genes present in the bone marrow implant. The HIF-1 can be injected either as the protein, or as the gene. If as the latter, it can be injected either in a plasmid or viral vector, or any other manner that leads to functionally relevant protein levels. For example, bone marrow can be transfected, ex vivo, with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes. It is emphasized, however, that HIF-1 is used in this section as an example of an intervention that could enhance production of angiogenic substances by bone marrow. This invention also covers use of other agents, which by enhancing HIF-1 activity (i.e., prolonging its half-life), or by producing effects analogous to HIF-1, stimulate the bone marrow to increase expression of angiogenic factors. A similar approach involves the exposure of autologous bone marrow to endothelial PAS domain protein 1 (EPAS1). EPAS1 shares high structural and functional homology with HIF-1.

Because VEGF promoter activity is enhanced by HIF-1, this invention also includes the ex-vivo exposure of bone marrow cells in culture to hypoxia or other forms of energy, such as, for example, ultrasound, RF, or electromagnetic energy. This intervention increases VEGF and other gene expression. By this effect it may augment the capacity of bone marrow to stimulate angiogenesis.

Another aspect of the invention involves the ex-vivo stimulation of aspirated autologous bone marrow by HIF-1 (or products that augment the effects of HIF-1 or produce similar effects to HIF-1 on bone marrow) or direct exposure of bone marrow to hypoxic environment followed by the delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e.g., ischemic limb) to enhance collateral-dependent perfusion in cardiac and/or peripheral ischemic tissue. The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant

genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes.

Current data indicate the importance of monocyte-derived cytokines for enhancing collateral function. Monocytes are activated during collateral growth in vivo, and monocyte chemoattractant protein-1 (MCP-1) is upregulated by shear stress in vitro. It has been shown that monocytes adhere to the vascular wall during collateral vessel growth (arteriogenesis) and capillary sprouting (angiogenesis). MCP-1 was also shown to enhance collateral growth after femoral artery occlusion in the rabbit chronic hindlimb ischemia model (Ito et al., *Circ Res* 1997; 80:829–3). Activation of monocytes seems to play an important role in collateral growth as well as in capillary sprouting. Increased monocyte recruitment by LPS is associated with increased capillary density as well as enhanced collateral and peripheral conductance at 7 days after experimental arterial occlusion (Arras M. et al., *J Clin Invest* 1998;101:40–50.)

A further aspect of the invention involves the ex-vivo stimulation of aspirated autologous bone marrow by MCP-1, followed by the direct delivery of activated bone marrow cells to the ischemic myocardium or peripheral organ (e.g., ischemic limb) to enhance collateral-dependent perfusion and muscular function in cardiac and/or peripheral ischemic tissue. The stimulation of the bone marrow could be by the direct exposure of the bone marrow to MCP-1 in the form of the protein, or the bone marrow cells can be transfected with a vector carrying the MCP-1 gene. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the MCP-1 transgene.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Granulocyte-Colony Stimulatory Factor (G-CSF) are stimulatory cytokines for monocyte maturation and are multipotent hematopoietic growth factors, which are utilized in clinical practice for various hematological pathologies such as depressed white blood cell count (i.e., leukopenia or granulocytopenia or monocytopenia) which occurs usually in response to immunosuppressive or chemotherapy treatment in cancer patients. GM-CSF has also been described as a multilineage growth factor that induces in vitro colony formation from erythroid burst-forming units, eosinophil colony-forming units (CSF), and multipotential (CSF), as well as from granulocyte-macrophage CSF and granulocyte CFU. (Bot F. J., *Exp Hematol* 1989, 17:292–5). Ex-vivo exposure to GM-CSF has been shown to induce rapid proliferation of CD-34+ progenitor cells (Egeland T. et al., *Blood* 1991; 78:3192–9.) These cells have the potential to differentiate into vascular endothelial cells and may naturally be involved in postnatal angiogenesis. In addition, GM-CSF carries multiple stimulatory effects on macrophage/monocyte proliferation, differentiation, motility and survival (reduced apoptotic rate). Consistent with the combined known effects on bone marrow derived endothelial progenitor cells and monocytes, it is another aspect of the invention to use GM-CSF as an adjunctive treatment to autologous bone marrow injections aimed to induce new blood vessel formation and differentiation in ischemic cardiovascular organs. Moreover, GM-CSF may further enhance therapeutic myocardial angiogenesis caused by bone marrow, by augmenting the effect of bone marrow, or by further stimulating, administered either in vivo or in vitro, bone marrow that is also being stimulated by agents such as HIF-1, EPAS1, hypoxia, or MCP-1.

In the examples below, certain testing regarding aspects of the invention is set forth. These examples are non-limitative.

EXAMPLES

Example 1

Effect of Bone Marrow Cultured Media on Endothelial Cell Proliferation

Studies were conducted to determine whether aspirated pig autologous bone marrow cells obtained secreted VEGF, a potent angiogenic factor, and MCP-1, which recently has been identified as an important angiogenic co-factor. Bone marrow was cultured *in vitro* for four weeks. The conditioned medium was added to cultured pig aortic endothelial cells (PAECs), and after four days proliferation was assessed. VEGF and MCP-1 levels in the conditioned medium were assayed using ELISA. During the four weeks in culture, BM cells secreted VEGF and MCP-1, such that their concentrations increased in a time-related manner. The resulting conditioned medium enhanced, in a dose-related manner, the proliferation of PAECs. The results indicate that BM cells are capable of secreting potent angiogenic cytokines such as VEGF and MCP-1 and of inducing proliferation of vascular endothelial cells.

Pig Bone Marrow Culture

Bone marrow (BM) cells were harvested under sterile conditions from pigs with chronic myocardial ischemia in preservative free heparin (20 units/ml BM cells) and filtered sequentially using 300 μ and 200 μ stainless steel mesh filters. BM cells were then isolated by Ficoll-Hypaque gradient centrifugation and cultured in long-term culture medium (LTCM) (Stem Cell Tech, Vancouver, British Columbia, Canada) at 33 $^{\circ}$ C. with 5% CO $_2$ in T-25 culture flask. The seeding density of the BMCs in each culture was 7 \times 10 6 /ml. Weekly, one half of the medium was removed and replaced with fresh LTCM. The removed medium was filtered (0.2 μ filter) and stored at -200 $^{\circ}$ C. for subsequent Enzyme-linked Immunosorbent Assay (ELISA) and cell proliferation assays.

Isolation and Culture of Pig Aortic Endothelial Cells

Fresh pig aortic endothelial cells (PAECs) were isolated using conventional methods. Endothelial cell growth medium (EGM-2 medium, Clonetics, San Diego, Calif.), containing 2% FBS, hydrocortisone, human FGF, VEGF, human EGF, IGF, heparin and antibiotics, at 37 $^{\circ}$ C. with 5% carbon dioxide. When the cells became confluent at about 7 days, they were split by 2.5% trypsin and cultured thereafter in medium 199 with 10% FBS. Their identity was confirmed by typical endothelial cell morphology and by immunohistochemistry staining for factor VIII. Passage 3-10 were used for the proliferation study.

Effects of Conditioned Medium on Aortic Endothelial Cells

Cell proliferation assay: PAECs (Passage 3-10) were removed from culture flasks by trypsinization. The detached cells were transferred to 96-well culture plates and plated at a seeding density of 5,000 cells/well. Cells were cultured for 2-3 days before being used in proliferation and DNA synthesis experiments. The conditioned medium of BM cells cultures were collected at 4 weeks; medium from 7 culture flasks were pooled and used in the bioassay. Aliquotes (10 μ L, 30 μ L, 100 μ L or 200 μ L) of pooled conditioned medium, or LTCM (200 μ L, as control), were added to confluent PAECs in 96-well plates in triplicate. Four days following culture with conditioned medium or control medium, the PAECs were trypsinized and counted using a cell counter (Coulter Counter Beckman Corporation, Miami Fla.).

Effects of Conditioned Medium on PAEC DNA Synthesis

Aliquotes (10 μ L, 30 μ L, 100 μ L or 200 μ L) of conditioned medium from pooled samples or control medium (LTCM, 200 μ L) were added to PAECs in 96-well plate (same seeding density as above) in triplicate. After 2 days, 1 μ Ci tritiated thymidine was added to each well. Forty-eight hours later, DNA in PAECs was harvested using a cell harvester (Mach III M Tomtec, Hamden, Conn.) and radioactivity was counted by liquid scintillation counter (Multi-detector Liquid Scintillation Luminescence Counter EG&G Wallac, Turku, Finland).

Determination of VEGF and MCP-1 in Conditioned Medium by ELISA VEGF

The concentration of VEGF in conditioned medium was measured using a sandwich ELISA kit (Chemicon International Inc., Temecula, Calif.). Briefly, a plate pre-coated with anti-human VEGF antibody was used to bind VEGF in the conditioned medium or to a known concentration of recombinant VEGF. The complex was detected by the biotinylated anti-VEGF antibody, which binds to the captured VEGF. The biotinylated VEGF antibody in turn was detected by streptavidin-alkaline phosphatase and color generating solution. The anti-human VEGF antibody cross-reacts with porcine VEGF.

Determination of MCP-1 in Conditioned Medium by ELISA

The concentration of MCP-1 in conditioned medium was assayed by sandwich enzyme immunoassay kit (R & D Systems, Minneapolis, Minn.): a plate pre-coated with anti human MCP-1 antibody was used to bind MCP-1 in the conditioned medium or to a known concentration of recombinant protein. The complex was detected by the biotinylated anti-MCP-1 antibody, which binds to the captured MCP-1. The biotinylated MCP-1 antibody in turn was detected by streptavidin-alkaline phosphatase and color generating solution. The anti-human MCP-1 antibody cross-reacts with porcine MCP-1.

Results

The BM conditioned medium collected at four weeks increased, in a dose-related manner, the proliferation of PAECs (FIG. 1). This was demonstrated by counting the number of cells directly and by measuring tritiated thymidine uptake ($p < 0.001$ for both measurements). The dose-related response demonstrated a descending limb; proliferation decreased with 200 μ L conditioned medium compared to 30 μ L and 100 μ L ($P = 0.003$ for both comparisons). Similar dose-related results were observed in the tritiated thymidine uptake studies ($P = 0.03$ for 30 μ L and 100 μ L compared to 200 μ L, respectively).

A limited number ($5 \pm 4\%$) of freshly aspirated BM cells stained positive for factor VIII. The results are set forth in FIG. 2. This contrasted to $57 \pm 14\%$ of the adherent layer of BM cells cultured for 4 weeks, of which $60 \pm 23\%$ were endothelial-like cells and $40 \pm 28\%$ appeared to be megakaryocytes.

Over a 4-week period, the concentrations of VEGF and MCP-1 in the BM conditioned medium increased gradually to 10 and 3 times the 1st week level, respectively ($P < 0.001$ for both comparisons) (FIG. 3). In comparison, VEGF and MCP-1 levels in a control culture medium, not exposed to BM, were 0 and 11 ± 2 pg/ml, respectively, as shown in FIG. 4.

Effects of Hypoxia on VEGF Secretion by Cultured Pig Bone Marrow Cells

It was demonstrated that hypoxia markedly increases the expression of VEGF by cultured bone marrow endothelial cells. results indicating that ex-vivo exposure to hypoxia, by increasing expression of hypoxia-inducible angiogenic factors, can further increase the collateral enhancing effect of bone marrow cells and its conditioned media to be injected in ischemic muscular tissue. Pig bone marrow was harvested and filtered sequentially using 300 μ and 200 μ stainless steel mesh filters. BMCs were then isolated by Ficoll-Hypaque gradient centrifugation and cultured at 33° C. with 5% CO₂ in T-75 culture flasks. When cells became confluent at about 7 days, they were split 1:3 by trypsinization. After 4 wks of culture, the BMCs were either exposed to hypoxic conditions (placed in a chamber containing 1% oxygen) for 24 to 120 hrs. or maintained under normal conditions. The resulting conditioned medium was collected and VEGF, MCP-1 were analyzed by ELISA.

Exposure to hypoxia markedly increased VEGF secretion: At 24 hrs VEGF concentration increased from 106 \pm 13 pg/ml under normoxic, to 1,600 \pm 196 pg/ml under hypoxic conditions ($p=0.0002$); after 120 hrs it increased from 4,163 \pm 62 to 6,028 \pm 167 pg/ml ($p<0.0001$). A separate study was performed on freshly isolated BMCs, and the same trend was found. Hypoxia also slowed the rate of proliferation of BMCs. MCP-1 expression was not increased by hypoxia, a not unexpected finding as its promoter is not known to have HIF binding sites.

Example 3

Effect of Bone Marrow Cultured Media on Endothelial Cell Tube Formation

It was demonstrated, using pig endothelial cells and vascular smooth muscle cells co-culture technique, that the conditioned medium of bone marrow cells induced the formation of structural vascular tubes in vitro. No such effect on vascular tube formation was observed without exposure to bone marrow conditioned medium. The results suggest that bone marrow cells and their secreted factors exert pro-angiogenic effects.

Example 4

The effect of Transendocardial Delivery of Autologous Bone Marrow on Collateral Perfusion and Regional Function in Chronic Myocardial Ischemia Model

Chronic myocardial ischemia was created in 14 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. Four weeks after implantation, 7 animals underwent transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and 7 control animals were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratio of ischemic/normal zone \times 100)

improved in ABM-treated pigs but not in controls (ABM: 95 \pm 13 vs 81 \pm 11 at rest, $P=0.017$; 85 \pm 19 vs 72 \pm 10 during adenosine, $P=0.046$; Controls: 86 \pm 14 vs 86 \pm 14 at rest, $P=NS$; 73 \pm 17 vs 72 \pm 14 during adenosine, $P=0.63$). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 83 \pm 21 vs 60 \pm 32 at rest, $P=0.04$; 91 \pm 44 vs 35 \pm 43 during pacing, $P=0.056$; Controls: 69 \pm 48 vs 64 \pm 46 at rest, $P=0.74$; 65 \pm 56 vs 37 \pm 56 during pacing, $P=0.23$).

The results indicate that catheter-based transendocardial injection of ABM can augment collateral perfusion and myocardial function in ischemic myocardium, findings suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

Fourteen specific-pathogen-free domestic pigs weighing approximately 70 kg were anesthetized, intubated, and received supplemental O₂ at 2 L/min as well as 1-2% isoflurane inhalation throughout the procedure. Arterial access was obtained via right femoral artery isolation and insertion of an 8 French sheath. The left circumflex artery was isolated through a left lateral thoracotomy and a metal encased ameroid constrictor was implanted at the very proximal part of the artery. Four weeks after the ameroid constrictor implantation all pigs underwent (1) a selective left and right coronary angiography for verification of ameroid occlusion and assessment of collateral flow; (2) transthoracic echocardiography studies; and (3) regional myocardial blood flow assessment.

Bone Marrow Aspiration and Preparation and Intramyocardial Injection

Immediately after completion of the baseline assessment, all animals underwent BM aspiration from the left femoral shaft using standard techniques. BM was aspirated from 2 sites (3 ml per site) using preservative free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow was immediately macro-filtered using 300 μ and 200 μ stainless steel filters, sequentially. Then, the bone marrow was injected using a trans-endocardial injection catheter into the myocardium in 12 sites (0.2 ml per injection site for total of 2.4. ml) directed to the ischemic myocardial territory and its borderline region.

Echocardiography Study

Transthoracic echocardiography images of short and long axis views at the mid-papillary muscle level were recorded in animals at baseline and during pacing, at baseline and during follow-up evaluation at four weeks after ABM implantation. Fractional shortening measurements were obtained by measuring the % wall thickening (end-systolic thickness minus end-diastolic thickness/end-diastolic thickness) \times 100. Those measurements were taken from the ischemic territory (lateral area) and remote territory (anterior-septal area). Subsequently, a temporary pacemaker electrode was inserted via a right femoral venous sheath and positioned in the right atrium. Animals were paced at 180/minute for 2 minutes and echocardiographic images were simultaneously recorded.

Regional Myocardial Blood Flow

Regional myocardial blood flow measurements were performed at rest and during maximal coronary vasodilation by use of multiple fluorescent colored microspheres (Interactive Medical Technologies, West Los Angeles, Calif.) and quantified by the reference sample technique (Heymann Mass., et al., *Prog Cardiovasc Dis* 1977;20:55-79). Fluorescent microspheres (0.8 ml, 5 \times 10⁶ microspheres/ml, 15 μ m diameter in a saline suspension with 0.01% Tween 80) were injected into the left atrium via a 6F Judkins left 3.5 diagnostic catheter. Maximal coronary vasodilation was induced by infusing adenosine at a constant rate of 140

$\mu\text{g/kg/min}$ (Fujisawa USA, Deerfield, Ill.) into the left femoral vein over a period of 6 minutes. During the last 2 minutes of the infusion, microsphere injection and blood reference withdrawal were undertaken in identical fashion to the rest study.

Following completion of the perfusion assessment, animals were sacrificed with an overdose of sodium pentobarbital and KCL. Hearts were harvested, flushed with Ringer Lactate, perfusion-fixed for 10–15 minutes, and subsequently immersion-fixed with 10% buffered formaldehyde for 3 days. After fixation was completed, the hearts were cut along the short axis into 7-mm thick slices. The 2 central slices were each divided into 8 similar sized wedges, which were further cut into endocardial and epicardial subsegments. The average of 8 lateral ischemic zone and 8 septal normal zone sub-segments measurements were used for assessment of endocardial and epicardial regional myocardial blood flow. The relative collateral flow was also computed as the ratio of the ischemic zone/non ischemic zone (IZ/NIZ) blood flow.

Histopathology

To assess whether injecting BM aspirate via the use of an injection catheter was associated with mechanical cell damage, standard BM smears were prepared before and after propelling the freshly filtered ABM aspirate through the needle using similar injecting pressure as in the in-vivo study. Morphological assessment was performed by an independent experienced technician who was blinded to the study protocol.

Histopathology assessment was performed on sampled heart tissue. In the pilot study, 7-mm thick short-axis slices were examined under UV light to identify fluorescent-tagged areas. Each identified area was cut into 3 full thickness adjacent blocks (central, right and left) that were immersion-fixed in 10% buffered formaldehyde. Subsequently, each such block was cut into 3 levels, of which 2 were stained with Hematoxylin and Eosin (H&E) and one with PAS. In addition, one fresh fluorescent-labeled tissue block was obtained from the ischemic region of each animal and was embedded in OCT compound (Sakura Finetek USA Inc., Torrance, Calif.) and frozen in liquid nitrogen. Frozen sections of these snap-frozen myocardial tissue were air dried and fixed with acetone. Immunoperoxidase stain was performed with the automated Dako immunostainer (Dako, Carpinteria, Calif.). The intrinsic peroxidase and non-specific uptake were blocked with 0.3% hydrogen peroxide and 10% ovo-albumin. Monoclonal mouse antibody against CD-34 (Becton Dickinson, San Jose, Calif.) was used as the primary antibody. The linking antibody was a biotinylated goat anti-mouse IgG antibody and the tertiary antibody was streptavidin conjugated with horse reddish peroxidase. Diaminobenzidine (DAB) was used as the chromogen and the sections were counterstained with 1% methylgreen. After dehydration and clearing, the slides were mounted and examined with a Nikon Labphot microscope.

In the efficacy study, full-thickness, 1.5 square centimeter sections from the ischemic and non-ischemic regions were processed for paraffin sections. Each of the samples was stained with H&E, Masson's trichrome, and factor VIII related antigen. The immunoperoxidase stained slides were studied for density of endothelial cell population and vascularization. The latter was distinguished from the former by the presence of a lumen. Vascularity was assessed using 5 photomicrographs samples of the factor VIII stained slides taken from the inner half of the ischemic and non-ischemic myocardium. Density of endothelial cells was assessed using digitized images of the same photomicrographs. The

density of the endothelial population was determined by Sigma-Scan Pro morphometry software using the intensity threshold method. The total endothelial area for each sample as well as for each specimen were obtained along with the relative percent endothelial area (endothelial area/area of the myocardium studied). The total endothelial area was also calculated as the relative percent of the non-infarcted (viable) area of the myocardium studied. The trichrom stained sections were digitized and the area occupied by the blue staining collagen as well as the total area of the section excluding the area occupied by the epicardium (which normally contained collagen) were measured using Sigma-Scan Pro. The infarcted area was then calculated as the area occupied by the blue staining.

Procedural Data

Intra-myocardial injections either with ABM or placebo were not associated with any acute change in mean blood pressure, heart rate or induction of arrhythmia. All hemodynamic parameters were comparable between the two groups. Pair-wise comparison showed similar hemodynamic parameters within each group in the index compared to the follow-up procedure except for higher initial mean arterial blood pressure at follow-up in the control group ($P=0.03$) with no subsequent differences during pacing or adenosine infusion.

Myocardial Function

Regional myocardial function assessment is shown in Table I below. Pre-intervention relative fractional wall thickening, expressed as ischemic zone to non-ischemic zone (IZ/NIZ) ratio $\times 100$, at rest and during pacing, was similar between groups ($P=0.86$ and 0.96 , respectively). At 4 weeks following the intra-myocardial injection of ABM, improved regional wall thickening occurred at rest and during pacing, which was due to an $\sim 50\%$ increase in wall thickening of the collateral-dependent ischemic lateral wall. No significant changes were observed in the control animals, although a trend towards improvement in wall thickening was noted in the ischemic area during pacing at follow-up.

TABLE I

	Regional Contractility of the Ischemic Wall		
	Baseline	Follow-up	P
Rest			
ABM (%)	60 \pm 32	83 \pm 21	0.04
Control (%)	64 \pm 46	69 \pm 48	0.74
Pacing			
ABM (%)	36 \pm 43	91 \pm 44	0.056
Control (%)	37 \pm 56	65 \pm 56	0.23

ABM indicates autologous bone marrow.

Myocardial Perfusion Data

Regional myocardial perfusion assessment is shown in Table II below. There were no differences between the treated and control groups in the pre-intervention relative transmural myocardial perfusion, IZ/NIZ, at rest and during adenosine infusion ($P=0.42$ and 0.96 , respectively). At 4 weeks following ABM injection, relative regional transmural myocardial perfusion at rest and during pacing improved significantly. This was due to an absolute improvement in myocardial perfusion in the ischemic zone both at rest (an increase of 57%, $P=0.08$) and during adenosine infusion (37%, $P=0.09$), while no significant changes were noted in absolute flow to the non-ischemic zone either at rest (increase of 35%, $P=0.18$) or during adenosine infusion (in-

crease of 25%, $P=0.26$). The increase in regional myocardial blood flow found in the ischemic zones consisted of both endocardial (73%) and epicardial (62%) regional improvement at rest, with somewhat lesser improvement during adenosine infusion (40% in both zones). At 4 weeks, the control group showed no differences in transmural, endocardial or epicardial perfusion in the ischemic and non-ischemic zones compared to pre-intervention values.

TABLE II

	<u>Regional Myocardial Perfusion</u>		
	Baseline	Follow-up	P
<u>Rest</u>			
ABM (%)	83 ± 12	98 ± 14	0.001
Control (%)	89 ± 9	92 ± 0.1	0.43
<u>Adenosine</u>			
ABM (%)	78 ± 12	89 ± 18	0.025
Control (%)	77 ± 5	78 ± 11	0.75

ABM indicates autologous bone marrow.

Histopathology and Vascularity Assessment

Assessment of BM smears before and after passing the filtrated aspirate through the injecting catheter revealed normal structure, absence of macro-aggregates and no evidence of cell fragments or distorted cell shapes. Histopathology at day 1 following injections revealed acute lesions characterized by fibrin and inflammatory tract with dispersed cellular infiltration. The infiltrate was characterized by mononuclear cells that morphologically could not be differentiated from a BM infiltrate. Cellularity was maximal at 3 and 7 days and declined subsequently over time. At 3 weeks, more fibrosis was seen in the 0.5 ml injection-sites compared to the 0.2 ml. CD-34 immunostaining, designed to identify BM-derived progenitor cells, was performed in sections demonstrating the maximal cellular infiltrate. Overall, it was estimated that 4-6% of the cellular infiltrate showed positive immunoreactivity to CD-34.

The ischemic territory in both groups was characterized by small areas of patchy necrosis occupying overall <10% of the examined ischemic myocardium. The non-ischemic area revealed normal myocardial structure. Changes in the histomorphometric characteristics of the two groups were compared. There were no differences in the total area occupied by any blood vessel as well as the number of blood vessels >50 μ m in diameter. However, comparison of the total areas stained positive for factor VIII (endothelial cells with and without lumen) in the ischemic versus the non-ischemic territories revealed differences between the 2 groups. In the ABM group, the total endothelial cell area in the ischemic collateral-dependent zone was 100% higher than that observed in the non-ischemic territory (11.6 ± 5.0 vs. $5.7 \pm 2.3\%$ area, $P=0.016$), whereas there was no significant difference in the control group (12.3 ± 5.5 vs. $8.2 \pm 3.1\%$ area, $P=0.11$). However, other parameters of vascularity, including % area occupied by any blood vessel and number of blood vessels >50 μ m were similar in the ischemic and non-ischemic territories in both groups.

Example 5

The Effect of Autologous Bone Marrow Stimulated in vivo by Pre-Administration of GM-CSF in Animal Model of Myocardial Ischemia

Chronic myocardial ischemia was created in 16 pigs by the implantation of ameroid constrictors around the left circumflex coronary artery. At four weeks minus 3 days after ameroid implantation, 8 animals underwent subcutaneous injection of GM-CSF for 3 consecutive days (dose 10 μ g/kg per day) followed (on the fourth day and exactly 4 weeks after ameroid implantation) by transendocardial injections of freshly aspirated ABM into the ischemic zone using a transendocardial injection catheter (2.4 ml per animal injected at 12 sites) and 8 control animals without GM-CSF stimulation were injected with heparinized saline. At baseline and 4 weeks later, animals underwent rest and pacing echocardiogram to assess regional contractility (% myocardial thickening), and microsphere study to assess collateral-dependent perfusion at rest and during adenosine infusion. Four weeks after injection of ABM collateral flow (expressed as the ratio of ischemic/normal zone $\times 100$) improved in ABM-treated pigs but not in controls (ABM: 85 ± 11 vs 72 ± 16 at rest, $P=0.026$; 83 ± 18 vs 64 ± 19 during adenosine, $P=0.06$; Controls: 93 ± 10 vs 89 ± 9 at rest, $P=0.31$; 73 ± 17 vs 75 ± 8 during adenosine, $P=0.74$). Similarly, contractility increased in ABM-treated pigs but not in controls (ABM: 93 ± 33 vs 63 ± 27 at rest, $P=0.009$; 84 ± 36 vs 51 ± 20 during pacing, $P=0.014$, Controls: 72 ± 45 vs 66 ± 43 at rest, $P=0.65$; 70 ± 36 vs 43 ± 55 during pacing, $P=0.18$).

The results indicate that catheter-based transendocardial injection of ABM pre-stimulated in vivo by GM-CSF administered systemically for 3 days, can augment collateral perfusion and myocardial function in ischemic myocardium, findings suggesting that this approach may constitute a novel therapeutic strategy for achieving optimal therapeutic angiogenesis.

Example 6

Treatment of a Human Patient

Bone marrow (~5 ml) will be aspirated from the iliac crest at approximately 60 minutes prior to initiation of the cardiac procedure using preservative-free heparinized glass syringes (20 unit heparin/1 ml fresh BM). The aspirated bone marrow will be immediately macro-filtered using 300 μ and 200 μ stainless steel filters, sequentially. An experienced hematologist will perform the procedure under sterile conditions. The bone marrow smear will be evaluated to confirm a normal histomorphology of the bone marrow preparation.

Any of several procedures for delivery of an agent to the myocardium can be used. These include direct transepical delivery, as could be achieved by a surgical approach (for example, but not limited to, a transthoracic incision or transthoracic insertion of a needle or other delivery device, or via thoracoscopy), or by any of several percutaneous procedures. Following is one example of percutaneous delivery. It should be emphasized that the following example is not meant to limit the options of delivery to the specific catheter-based platform system described in the example—any catheter-based platform system can be used.

Using standard procedures for percutaneous coronary angioplasty, an introducer sheath of at least 8F is inserted in the right or left femoral artery. Following insertion of the arterial sheath, heparin is administered and supplemented as

needed to maintain an ACT for 200–250 seconds throughout the LV mapping and ABM transplantation portion of the procedure. ACT will be checked during the procedure at intervals of no longer than 30 minutes, as well as at the end of the procedure to verify conformity with this requirement.

Left ventriculography is performed in standard RAO and/or LAO views to assist with guidance of NOGA-STAR™ and injection catheters, and an LV electromechanical map is obtained using the NOGA-STAR™ catheter. The 8F INJECTION-STAR catheter is placed in a retrograde fashion via the femoral sheath to the aortic valve. After full tip deflection, the rounded distal tip is gently prolapsed across the aortic valve and straightened appropriately once within the LV cavity.

The catheter (incorporating an electromagnetic tip sensor) is oriented to one of the treatment zones (e.g. anterior, lateral, inferior-posterior or other). Utilizing the safety features of the NOGA™ system, needle insertion and injection is allowed only when stability signals will demonstrate an LS value of <3. A single injection of 0.2 cc of freshly aspirated ABM will be delivered via trans-endocardial approach to the confines of up to two treatment zones with no closer than 5 mm between each injection site. The density of injection sites will depend upon the individual subject's LV endomyocardial anatomy and the ability to achieve a stable position on the endocardial surface without catheter displacement or premature ventricular contractions (PVCs).

That freshly aspirated autologous bone marrow transplanted into ischemic myocardium is associated with improved collateral flow without adverse effects may be of clinical importance for several reasons. The methodology reflected above took advantage of the natural capability of the bone marrow to induce a localized angiogenic response in an effective and apparently safe manner. Such an angiogenic strategy would probably be less costly than many others currently being tested. It would also avoid potential toxicity-related issues that are remote but definite possibilities with various gene-based approaches using viral vectors.

The invention is based on the concept that autologous bone marrow may be an optimal source for cellular (an example would be endothelial progenitor cells, but the invention is not limited to such cells as many other cells in the bone marrow may contribute importantly to the angiogenic effect) and secreted, e.g., angiogenic growth factors, elements necessary to promote new blood vessel growth and restore function when transferred to another tissue, such as ischemic heart or peripheral limbs. A patient's own bone marrow can be used as the key therapeutic source to induce therapeutic angiogenesis and/or myogenesis in ischemic tissues, e.g., heart muscle and/or ischemic limb, with compromised blood perfusion due to arterial obstructions. The patient's own bone marrow is aspirated, i.e., autologous bone marrow donation, processed, and injected directly into ischemia and/or adjacent non-ischemic tissue, e.g., heart muscle and/or ischemic limb, to promote blood vessel growth.

The autologous bone marrow and/or bone marrow products are injected into the heart muscle, e.g., the myocardium, by use of either a catheter-based trans-endocardial injection approach or a surgical (open chest or via thoracoscopy) trans-epicardial thoracotomy approach. Those two delivery strategies can be used to achieve the same therapeutic goal by promoting the incorporation and integration of angiogenic bone marrow elements in the target organ tissue, e.g., heart muscle and/or ischemic limb.

According to the invention, effective amounts of autologous bone marrow are administered for treatment. As would

be appreciated by experienced practitioners, the amount administered will depend upon many factors, including, but not limited to, the intended treatment, the severity of a condition being treated, the size and extent of an area to be treated, etc. With regard to treatment according to the invention, a representative protocol would be to administer quantities of from about 0.2 to about 0.5 ml of autologous bone marrow in each of from about 12 to about 25 injections, for a total of from about 2.4 to about 6 ml of autologous bone marrow being administered. Each dose administered could preferably comprise from about 1 to about 2 percent by volume of heparin or another blood anticoagulant, such as coumadin. When the autologous bone marrow has been cultured or stimulated and/or is being administered in combination with other pharmaceuticals or the like, the quantity of autologous bone marrow present should be approximately the same in each dose and/or the total of the autologous bone marrow administered should be about the same as described above. It is believed that the total number of cells of autologous bone marrow administered in each treatment should be on the order of from about 10^7 to 5×10^8 .

Optimization of angiogenic gene expression requires the co-administration of various angiogenic stimulants with the autologous bone marrow. Thus, according to the invention autologous bone marrow transplantation is injected either as a "stand alone" therapeutic agent or combined with any pharmacologic drug, protein or gene or any other compound or intervention that may enhance bone marrow production of angiogenic growth factors and/or promote endothelial cell proliferation, migration, and blood vessel tube formation. The "combined" agent(s) can be administered directly into the patient or target tissue, or incubated ex-vivo with bone marrow prior to injection of bone marrow into the patient. Examples of these "combined" agents (although not limited to these agents) are Granulocyte-Monocyte Colony Stimulatory Factor (GM-CSF), Monocyte Chemoattractant Protein 1 (MCP 1), EPAS1, or Hypoxia Inducible Factor-1 (HIF-1). The stimulation of the bone marrow could be by the direct exposure of the bone marrow to the factors in the form of proteins, or the bone marrow cells can be transfected with vectors carrying the relevant genes. For example, bone marrow can be transfected with a plasmid vector, or with an adenoviral vector, carrying the HIF-1 or EPAS1 transgenes. An example of an intervention that may enhance bone production of angiogenic factors is ex-vivo exposure of bone marrow cells to hypoxia. This intervention can be used alone with bone marrow, or in combination with any of the factors outlined above. These optimization strategies are designed to increase the production of vascular endothelial growth factor (VEGF) expression and/or other cytokines with angiogenic activity prior to the direct injection of the bone marrow into the heart or any peripheral ischemic tissue. In a broad sense, the invention comprises intramyocardial injection of autologous bone marrow with any agent that would become available to cause stimulation of bone marrow and/or ex-vivo or in vivo stimulation of any angiogenic growth factor production by the bone marrow or its stromal microenvironment.

Delivery to patients will vary, dependent upon the clinical situation. For example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy who will be candidates for a bone marrow aspiration procedure followed by autologous bone marrow myocardial or limb transplantation directed into the ischemic tissue or its borderline zone and/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogenesis and/or myogenesis. For

example, patients with refractory coronary artery disease or ischemic peripheral vasculopathy who will be candidates for a bone marrow aspiration procedure followed by autologous bone marrow myocardial or limb transplantation directed into the ischemic tissue or its borderline zone and/or normal tissue that may serve as the source for collateral or cellular supply to the diseased tissue for the purposes of therapeutic angiogenesis and/or myogenesis. This procedure will involve the use of a bone marrow aspiration procedure, bone marrow harvesting and processing, followed by the use of the autologous bone marrow or its elements (growth factors and/or cellular elements being isolated from the patient's own bone marrow), with or without any ex-vivo stimulation of its delivery forms, to be injected into the ischemic or non ischemic myocardium and/or peripheral ischemic tissue (such as limb ischemia). The bone marrow will be kept in standard anti-coagulation/anti-aggregation solution (containing sodium citrate and EDTA) and kept in 4° C. in sterile medium until the time of its use.

Upon its use, the bone marrow will be filtered to avoid injecting remaining blood clots or macroaggregates into the target tissue.

The bone marrow, with or without a stimulatory agent in any of its delivery forms, or with or without having been transfected with a vector carrying a transgene that is designed to enhance the angiogenesis effect of the bone marrow, will be injected into the heart muscle, i.e., in therapeutic myocardial angiogenesis or therapeutic myogenesis, using either any catheter-based trans-endocardial injection device or via a surgical (open chest) trans-epicardial thoracotomy approach, or any other approach that allows for transepical delivery. In the case of treatment of limb ischemia the bone marrow will be transferred by a direct injection of the bone marrow or its elements, with or without ex-vivo or in vivo stimulation in any of its delivery forms, into the muscles of the leg.

The volume of injection per treatment site will probably range between 0.1-5.0 cc per injection site, dependent upon the specific bone marrow product and severity of the ischemic condition and the site of injection. The total number of injections will probably range between 1-50 injection sites per treatment session.

The preceding specific embodiments are illustrative of the practice of the invention. It is to be understood, however, that other expedients known to those skilled in the art or disclosed herein, may be employed without departing from the spirit of the invention or the scope of the appended claims.

We claim:

1. A method of enhancing collateral blood vessel formation in a subject comprising directly administering to sites in heart or limb tissue an effective amount of autologous bone marrow aspirate to induce collateral blood vessel formation in the tissue.

2. The method of claim 1, wherein the autologous bone marrow aspirate is injected.

3. The method of claim 1, wherein the autologous bone marrow aspirate is injected intramyocardially.

4. The method of claim 2 wherein the wherein the autologous bone marrow aspirate is injected trans-epicardially or trans-endocardially.

5. The method of claim 4, wherein the trans-endocardial approach is via a catheter.

6. The method of claim 1, wherein the autologous bone marrow aspirate has been stimulated while growing in conditioned medium ex vivo, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

7. The method of claim 6, wherein the autologous bone marrow aspirate has been stimulated by contact with one or more angiogenesis stimulating cytokines secreted therefrom while growing in conditioned medium ex vivo, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

8. The method of claim 1, wherein the autologous bone marrow aspirate further comprises Monocyte Chemoattractant Protein 1 (MCP-1) or Vascular Endothelial Growth Factor (VEGF).

9. The method of claim 6, wherein the autologous bone marrow aspirate has been stimulated ex vivo in culture by transient exposure to hypoxia.

10. The method of claim 1, wherein the autologous bone marrow aspirate is administered in combination with one or more agent selected from a pharmacological drug or protein that enhances bone marrow production of angiogenic growth factors selected to promote endothelial cell proliferation, migration, or blood vessel formation.

11. The method of claim 10, wherein the autologous bone marrow aspirate and the agent or agents are administered together.

12. The method of claim 10, wherein the autologous bone marrow aspirate and the agent or agents are combined ex vivo prior to administration.

13. The method of claim 12, wherein the autologous bone marrow aspirate has been stimulated ex vivo in conditioned medium, the conditioned medium comprising at least one agent selected from granulocyte-monocyte colony stimulating factor (GM-CSF), endothelial PAS domain 1 (EPAS1) and hypoxia inducible factor 1 (HIF-1).

14. The method of claim 1, wherein the autologous bone marrow aspirate is administered to ischemic tissue.

15. The method of claim 12, further comprising culturing the autologous bone marrow aspirate to form conditioned medium containing bone marrow cells and endogenously secreted angiogenic cytokines and injecting the composition into ischemic heart tissue.

* * * * *

EVIDENCE APPENDIX

ITEM NO. 18

2004 Rabelink et al. publication in Arteriosclerosis, Thrombosis, and Vascular Biology, entitled, “Endothelial Progenitor Cells: More Than an Inflammatory Response?” cited by the Examiner in the October 2, 2008 Office Action

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Endothelial Progenitor Cells: More Than an Inflammatory Response?

Ton J. Rabelink, Hetty C. de Boer, Eelco J.P. de Koning, Anton-Jan van Zonneveld

Abstract—The formation of new capillaries (angiogenesis) may be of clinical importance in facilitating reperfusion and regeneration of hibernating cardiac tissue after myocardial infarction and in microvascular ischemia. Evidence is accumulating that as part of the response to hypoxia, bone marrow-derived circulating endothelial progenitor cells (CEPs) are mobilized and subsequently differentiate into proper endothelial cells. There are also indications that such CEPs can facilitate endothelial repair and angiogenesis in vivo. It is not clear yet, however, whether these CEPs are essential for these adaptive processes or what the relative contribution of CEP is compared with that of other mononuclear inflammatory cells that are mobilized to areas of ischemia. Moreover, there are still many uncertainties about how cardiovascular risk factors alter CEP function. Particularly when therapeutically mobilizing CEPs, a further understanding of this issue is essential to assess the risk of potentially harmful side effects of altered CEP function. (*Arterioscler Thromb Vasc Biol.* 2004;24:834-838.)

Key Words: angiogenesis ■ atherosclerosis ■ endothelial progenitor

Over the past 7 years, the discovery of the phenomenon that mononuclear cells in peripheral blood have the potential to differentiate into endothelial cells ex vivo as well as in vivo has opened up a new field of cardiovascular research.^{1,2} It thus appears that such endothelial progenitor cells (EPCs) can be used therapeutically to restore damaged endothelium.^{3,4} They can also incorporate into the endothelial monolayer and stimulate proliferation of neighboring endothelial cells, thus inducing the formation of new blood vessels.^{5,6} Although the biology is not really understood, several pilot studies have suggested beneficial effects of infusion of mononuclear cells after myocardial infarction in animal models⁷⁻¹⁰ and in humans.¹¹ The aim of the current review is to discuss some of the potential regulatory mechanisms involved in this phenomenon. In particular, we address the question of whether these progenitor cells are a specific subpopulation with stem cell properties or whether these cells merely reflect plasticity of the normal inflammatory response that occurs in occlusive vascular disease. Ultimately, understanding this biology will be a critical success factor for bringing progenitor cell therapy into the clinical arena.

Vascular Occlusion Leads to Inflammation

In the adult, vessels grow either via capillary sprouting (angiogenesis) or via remodeling of pre-existing arteriolar connections into collateral vessels (arteriogenesis).^{12,13} Both processes occur on occlusion of a vessel, thus improving blood delivery and local perfusion of ischemic tissue. The regulation of the cellular processes involved in arteriogenesis

has recently been reviewed in this journal.¹³ In this review, we focus on the contribution of bone marrow-derived progenitor cells in the (microcirculatory) angiogenic response to ischemia, a process referred to as (postnatal) vasculogenesis. The endothelial cell is key in initiating vasculogenesis. First, on vascular occlusion, endothelial cells will sense altered shear stress. Recent studies have shown that at low shear stress or oscillatory shear stress, endothelial cells typically will increase the expression of pro-oxidant enzymes, such as NADH oxidase,¹⁴ and reduce the expression of anti-oxidant enzymes such as manganese superoxide dismutase,¹⁵ thioredoxin reductase, and glutathione reductase.¹⁶ As a result, altered shear stress during vascular occlusion will result locally in increased redox signaling.¹⁷ One of the activated transcription factors is NF- κ B, which plays a central role in the inflammatory response.¹⁸ NF- κ B activation in the endothelium results in the expression of adhesion molecules and the release of chemotactic factors for inflammatory cells.¹⁹ In agreement, in situ nuclear translocation of NF- κ B has been found in the vessel wall near regions of disturbed blood flow, like bifurcations, curvatures, and branching points.²⁰ The second parameter that endothelial cells sense during vascular occlusion is hypoxia. Basically, the endothelial cell is equipped with 2 systems to sense such hypoxia. One is the transcription factor HIF-1 α .²¹ Under normoxic conditions, this transcription factor will be hydroxylated on a conserved prolyl residue in a reaction with molecular oxygen. The hydroxylated prolyl group allows the Von Hippel-Lindau protein to bind and polyubiquitate the molecule, thereby

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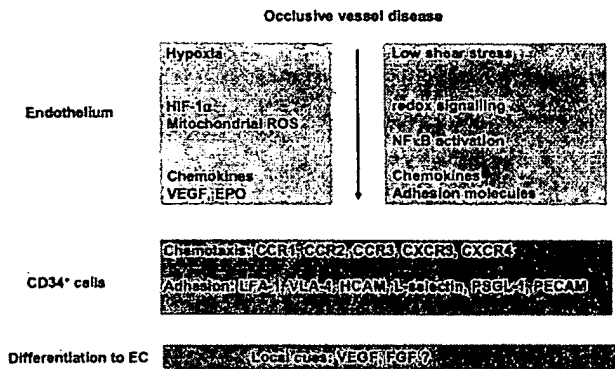


Figure 1. In atherosclerotic vascular disease, the endothelial cells will be exposed to hypoxia and altered shear stress. Both mechanisms induce redox signaling in the endothelial cell, with subsequent release of chemokines and surface expression of leukocyte adhesion molecules. The CD34⁺ hematopoietic stem cell is equipped with several receptors, which allow for chemotaxis, and with ligands for adhesion. Local cues such as the concentration of VEGF and basic FGF probably determine differentiation into endothelial-like cells.

emarking HIF-1 α for destruction by the proteasome. However, in hypoxia, this hydroxyproline is reduced, allowing HIF-1 α to accumulate and translocate to the nucleus to activate transcription of genes such as VEGF, EPO, and inducible NOS. The other oxygen-sensing mechanism in the endothelial cell is located in the mitochondria where, under normoxic conditions, oxidative phosphorylation occurs.²² This reaction is constitutively inhibited by nitric oxide that is also produced in the mitochondria. When hypoxia occurs, nitric oxide starts to dominate and reduces electron transport toward its final acceptor, complex IV.²³ As a result, intermediate radical products such as hydrogen peroxide and reactive nitrogen species are produced by the mitochondria, again leading to redox signaling and subsequent activation of the endothelial cell. Taken together, altered shear stress and hypoxia will result in redox-mediated endothelial cell activation and release of chemokines, cytokines, and growth factors. Thus, occlusive vascular disease inevitably results in a local inflammatory response of the endothelium (Figure 1).

Endothelial Progenitor Cells: Part of the Inflammatory Response?

For some time, it has been suggested that as a consequence of such an inflammatory response, the ensuing recruitment of monocytes are instrumental not only in inducing collateral formation but also in promoting angiogenesis.^{13,24} It is believed that paracrine release of cytokines and growth factors with known angiogenic properties, such as bFGF and TNF α , mediate these effects of monocytes on capillary sprouting.²⁵

More recently, attention has also been drawn to other vasculogenic cell populations present in the mononuclear fraction of peripheral blood that may also be recruited to the activated endothelium in response to an ischemic insult. These are referred to as pluripotent stem cell or progenitor cell populations and include the CD34⁺ hematopoietic stem cells and subpopulations of CD34⁺ mononuclear cells, and even subpopulations of the peripheral blood monocytes. Isolation of each of these subpopulations and subsequent

culture *in vitro* could give rise not only to classical circulating blood cells such as monocytes/macrophages but also to unexpected phenotypes such as endothelial cells and myocytes.^{1,26–28} Hence, the concept has evolved that vascular progenitors are recruited from the bone marrow to sites of tissue revascularization, where they participate in a paracrine way and also directly by differentiating into mature endothelial cells. In particular, the CD34⁺ hematopoietic stem cell has raised a lot of attention in this respect because of the similarities with the embryonic hemangioblast, which gives rise not only to circulating blood cell lineages but also to vascular cells.²⁹ Hematopoietic stem cells appear to be essential for angiogenesis in the mouse embryo³⁰ and, when durably engrafted in adult mice, were shown to have functional hemangioblast activity and develop into endothelial cells that participate in the neovasculature that evolved after retinal ischemia.³¹

Although bone marrow transplantation experiments have shown unequivocally that bone marrow-derived cells can also differentiate into vascular cells *in situ*, the frequency of this phenomenon and the identification of the cell type involved are still matter of debate.³² Only recently have some specific surface markers for “true” EPCs emerged from detailed studies characterizing mammalian embryogenesis and angiogenesis. Flk-1/KDR is a receptor for vascular endothelial cell growth factor (VEGFR-2), which appears to be critical for embryonic endothelial cell differentiation and vasculogenesis.³³ Also, it was reported that Flk-1–positive cells, derived from differentiated embryonic stem cells, can give rise to endothelial cells and vascular smooth muscle cells *in vitro* and *in vivo*.³⁴

Together with the essential role of Flk-1 in hematopoiesis,³⁵ these observations are consistent with the existence of a Flk-1–positive hemangioblast that serves as a common origin of endothelial cells and blood cells.³⁶ AC133 is a second early hematopoietic stem cell marker that is downregulated on differentiation and is therefore a marker for early EPCs. Indeed, AC133-positive cells from human peripheral blood were shown to differentiate into endothelial cells *in vitro*.³⁷ Using these stem cell markers, it has become clear that only a very small subset of circulating mononuclear cells in peripheral human blood stains (0.002%) positively for CD34, AC133, and Flk-1 simultaneously.³⁸ The most detailed phenotypic description of the circulating EPC (CEP) proposes the co-expression of several common endothelial and hematopoietic antigens: CD34⁺, FGFR⁺, CD38⁺, VE-cadherin⁺, c-kit⁺, CD31⁺, Flt-1, AC133⁺; in addition, it represents even a subfraction of these.³⁹

Are these CEP part of the inflammatory response on vascular occlusion and, if so, does contribution of these cells matter in view of the reported effects of the abundantly present monocytes on these processes? To play such a role, CEP should have the capacity to home exclusively on sites of angiogenesis. They should be able to attach to activated endothelium or extracellular matrix, to (trans)differentiate into an endothelial phenotype, and be able to proliferate. We recently demonstrated that CD34⁺ hematopoietic stem cells specifically home and migrate to angiogenic endothelium (unpublished observation). Although CD34⁺ cells probably

do not adhere to normal endothelium, they can attach to activated endothelium. Platelets may play an important modulating role in this attachment. Platelets can adhere to inflamed endothelial cells or to exposed extracellular matrix, where they express P-selectin and thus can provide an adhesive surface for CEP;⁴⁰ however, CD34⁺ cells express the binding determinant for P-selectin (PSGL-1).⁴¹ In particular, the issue whether endothelial progenitor cells can proliferate after homing and (trans)differentiation may be important in appreciating the *in vivo* relevance of these CEP as sources of paracrine factors and as sources for endothelial cells versus the effects that other inflammatory cells have on resident endothelium. Bone marrow transplantation experiments show low to very low percentages of *in situ* differentiation of bone marrow-derived cells into endothelial cells, making the role for CEPs as a major source of endothelial cells in the short-term perspective of these experiments less likely. However, in acute ischemic events such as myocardial infarction, the number of circulating CD34⁺ cells was increased and a direct correlation with plasma levels of VEGF was shown.⁴² Furthermore, in patients experiencing an acute vascular insult secondary to burns or coronary bypass grafting, a rapid increase (50×) in the number of CEP was noted within 6 to 12 hours after injury, which coincided with an elevation in VEGF level.⁴³ Clearly, increased numbers of circulating CD34⁺ cells, in combination with efficient homing and ultimately (trans)differentiation and proliferation at the site of vascular injury, may increase the contribution of these progenitor cells relative to other inflammatory cells. This phenomenon can be used therapeutically by artificially increasing the number of circulating progenitor cells in conditions such as ischemia. VEGF, basic fibroblast growth factor, angiopoietin-1, placental growth factor, and stromal cell-derived growth factor-1 have all been shown to induce EPC mobilization and recruitment.^{2,44} What most of these factors have in common is that they stimulate the Akt/PKB pathway; evidence is accumulating that the Akt/PKB pathway plays a central role in stem cell recruitment and survival.⁴⁵ Activation of this pathway may also explain some of the effect of statins on *in vivo* (re)endothelialization, an effect that appears, at least partly, dependent on incorporation of bone marrow-derived cells into the endothelial cell monolayer.^{4,46} Because EPCs are thought to be derived from the CD34⁺ hematopoietic stem cell, an additional method to increase circulating EPCs is to use stem cell mobilizing factors, such as stem cell factor and granulocyte-macrophage colony-stimulating factor. In experimental myocardial infarction^{47,48} and the ischemic hindlimb,⁴⁹ it was found that during therapy with such cytokines, circulating EPCs were mobilized into the ischemic regions and augmented neovascularization of ischemic tissue. Recently, a stimulatory effect of erythropoietin (Epo) has also been described on EPC recruitment and angiogenesis in the mouse model of inflammation- and ischemia-induced neovascularization.⁵⁰ Also, in renal anemia patients, recombinant Epo markedly increased the number of CD34⁺ cells in the circulation.⁵¹ Although these observations are exciting, it should be noticed that the beneficial effects of mobilizing CEPs on ischemia were observed in otherwise healthy animals. In disease states or in

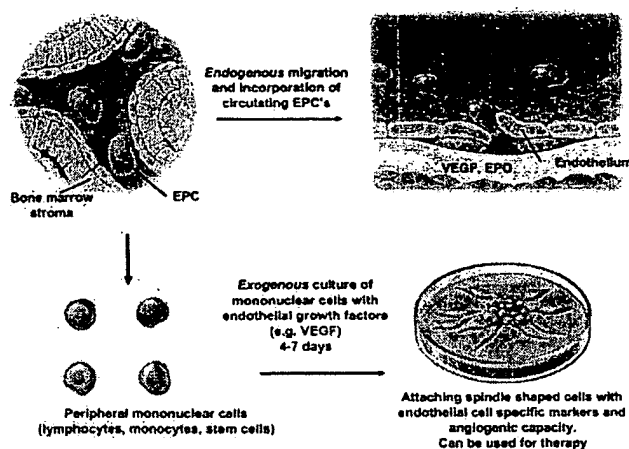


Figure 2. Endothelial progenitor cells are referred to as the endogenous CD34⁺-derived cells that can incorporate in damaged endothelium or hypoxic tissue (upper part of the figure) as well as the *ex vivo* expanded and differentiated mononuclear cells, which have been shown to display endothelial-specific cell markers and angiogenic capacity (lower part of the figure).

the presence of cardiovascular risk factors, mobilization of such cells may also promote the formation of potentially harmful CD34⁺-derived phenotypes such as macrophages or fibrocytes.⁵² It thus appears that cardiovascular risk factors may shift the balance between cells that can induce repair and neovascularization toward cells that contribute to a harmful inflammatory reaction. In addition, nitric oxide availability appears to be essential for mobilization of circulating EPCs from the bone marrow stroma.⁵³ Strategies that allow the beneficial side of inflammation such as the endogenous capacity to form endothelial-like cells while at the same time reducing differentiation of harmful cellular phenotypes by drugs that enhance nitric oxide bioavailability or activate the Akt/PKB signaling pathway (eg, statin therapy^{54,55}) may therefore prove to be even more useful than mobilizing or infusing progenitor cells.

Attaching Cells: The Other EPC

The low numbers of CD34⁺ CEP (100 to 500 per mL blood) are in sharp contrast with the relatively large numbers of attached cells that are obtained ($\approx 100\,000$ from 1 mL blood) after culturing the blood mononuclear cell fraction on fibronectin or gelatin for 4 days in the presence of endothelial growth factors and that, unfortunately, often also are referred to as "EPC" (Figure 2). These spindle-shaped attaching cells (hereafter referred to as AT cells) exhibit endothelial characteristics such as the potential to take-up acetylated LDL and expression of endothelial markers such as ULEX and von Willebrand factor.^{1,42} This remarkable plasticity of cells present in the AT cell cultures cannot be explained by the presence of a few co-isolated CEPs and more likely originate from a more abundant circulating mononuclear cell type, such as monocytes.^{26,56–59} The concept that has developed over the years is that the number of these AT cells quantitatively reflect subpopulations within the blood mononuclear cells that have the potential to differentiate into an endothelial phenotype *in vivo*. Interestingly, the number of AT cells is reduced in patients with cardiovascular risk factors.⁶⁰ Recently, this reduction has been related to intermedi-

ate endpoints of cardiovascular disease, such as impaired flow-mediated dilation.⁶¹ However, one has to realize that the AT cell cultures are an in vitro phenomenon and thus subject to methodological influences. For example, increased expression of matrix adhesion molecules such as the vitronectin receptor $\alpha_3\beta_3$ in mononuclear cells (eg, by statin therapy) may yield higher numbers of AT cells in culture conditions in which vitronectin is used as an adhesive surface.^{4,62} Thus, when certain adhesion receptors are altered in mononuclear cells, either disease- or therapy-related, then this will be noticed only when the mononuclear cells are cultured on the relevant adhesive protein(s). To date, ≈ 6 different adhesive surfaces have been used in the EPC culture assay: FN, FN plus gelatin, gelatin, VN plus gelatin, FN plus collagen, and collagen type I. To what extent these different surfaces have led to conflicting interpretations is not clear. However, we feel that the concept that the number of AT cells quantitatively reflects the number of circulating EPCs has to be carefully interpreted. Nevertheless, these AT cells appear to offer spectacular therapeutic opportunities. Intravenous infusion of these AT cells in animal models of ischemia results in homing of these cells to the ischemic tissue and augmentation of neovascularization.^{5,9,63} These effects are specific, because infusion with mature endothelial cells had no such an effect.⁵ Based on these observations, clinical studies have been initiated such as the TOP-CARE study, to investigate whether infusion of autologous expanded AT cells in patients with myocardial infarction reduces ischemic injury.¹¹

In conclusion, evidence is accumulating that, as part of the response to hypoxia, circulating endothelial progenitor cells are mobilized from the bone marrow and subsequently differentiate into proper endothelial cells. There are also indications that such CEPs can facilitate endothelial repair and angiogenesis in vivo. It is not clear yet, however, whether CEPs are essential for these adaptive processes or what the relative contribution of CEP is compared with that of other mononuclear inflammatory cells. Moreover, there are still many uncertainties about how cardiovascular risk factors modulate CEP function. Particularly when therapeutically mobilizing CEPs, a further understanding of this issue is essential to assess the risk of transdifferentiation of CEPs to potentially pro-atherogenic inflammatory cells.^{64,65}

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EVIDENCE APPENDIX

ITEM NO. 19

**Caplan 1991 publication in Journal of Orthopaedic Research,
entitled, “Mesenchymal Stem Cells” cited by Appellant as
Exhibit E in the Amendment filed June 26, 2006**

Mesenchymal Stem Cells*

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Summary: Bone and cartilage formation in the embryo and repair and turnover in the adult involve the progeny of a small number of cells called mesenchymal stem cells. These cells divide, and their progeny become committed to a specific and distinctive phenotypic pathway, a lineage with discrete steps and, finally, end-stage cells involved with fabrication of a unique tissue type, e.g., cartilage or bone. Local cuing (extrinsic factors) and the genomic potential (intrinsic factors) interact at each lineage step to control the rate and characteristic phenotype of the cells in the emerging tissue. The study of these mesenchymal stem cells, whether isolated from embryos or adults, provides the basis for the emergence of a new therapeutic technology of self-cell repair. The isolation, mitotic expansion, and site-directed delivery of autologous stem cells can govern the rapid and specific repair of skeletal tissues. **Key Words:** Mesenchymal stem cells—Bone—Cartilage—Differentiation—Self-cell therapy—Skeletal tissue—Embryo—Adult.

THE CONCEPT

It is generally agreed that in an embryo a mesenchymal stem cell is a pluripotent progenitor cell which divides many times and whose progeny eventually gives rise to skeletal tissues: cartilage, bone, tendon, ligament, marrow stroma, connective tissue (Fig. 1). By definition, these stem cells are not governed by or limited to a fixed number of mitotic divisions. Their progeny are affected by a number of factors, however, as they become tracked into very specific developmental pathways in which both intrinsic and extrinsic factors combine to control the molecular and cellular pattern of expression that results in specific tissues that perform specific functions based on their molecular repertoire (9,11).

Indeed, the progression from stem cell to final end phenotype is marked by discrete stages with transit from one stage to the next dependent on local cuing from surrounding cells (paracrine regulation) as well as signals emitted by the cell itself and the reception of its own signaling (autocrine regulation) (10,57). The sum of these various intrinsic and extrinsic signals defines the developmental position of the cells. Although difficult to reconstruct on a cell culture dish, such "positional information" has been experimentally approached by studying embryonic cells in culture, cells that have the potential to differentiate into various phenotypes (7,9,11,15).

The concept of stem cells is now well established (21,60). Two systems serve as models for such a concept: First, *Caenorhabditis elegans* is a small worm whose entire developmental lineage map has been described (21); every cell found in the adult has been carefully tracked and its progenitor tree precisely established with every branch and sub-branch delineated. Second, and to be emphasized, the hematopoietic cell lineage has been described with its several diverging pathways (21,52). It is now clear that each separate pathway and, indeed,

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* This publication was, in part, the basis for Dr. Caplan's receiving the Elizabeth Winston Lanier Award given by the American Academy of Orthopaedic Surgeons as part of their 1990 Kappa Delta Awards.

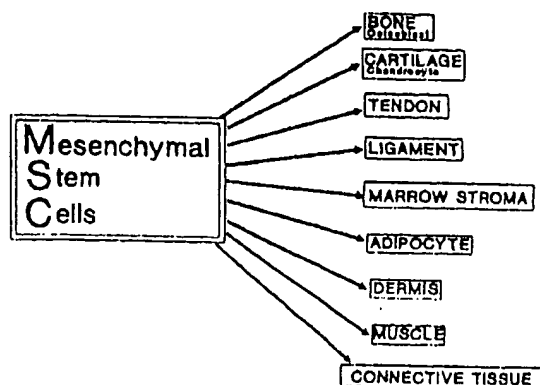


FIG. 1. Mesenchymal stem cell phenotypes. Mesenchymal stem cells are theoretically capable of differentiating through a series of separate and unique lineage transitions into a variety of end-stage phenotypes as shown.

progression through each separate stage within a discrete pathway is controlled by a balance of extrinsic and intrinsic macromolecules. Molecular biologists continue to isolate, clone, and express large amounts of these proteins, which allows use of cell culture systems to identify accurately the factor that controls progression to which stage and when (51,70). The challenge for skeletal biologists is to use the new information and new molecular tools to translate these advances into a better understanding of skeletal development, physiology, and repair.

EMBRYONIC MESENCHYMAL CELLS

The middle embryonic layer, the mesoderm, gives rise to all of the body's skeletal elements.* The term, mesenchyme, is derived from the Greek meaning "middle" (meso) "infusion" and refers to the ability of mesenchymatous cells to spread and migrate in early embryonic development between the ectodermal and endodermal layers. This characteristic migratory, space-filling ability is the key element of all wound repair in adult organisms involving mesenchymal cells in skin (dermis), bone (periosteum), or muscle (perimysium). Proteins that serve as chemoattractants, chemicals that specifically encourage this migratory activity to wound or developmental sites have been identified (24,32,59). The migratory activity of mesenchymal cells is complemented by their capacity to aggregate spe-

* For the sake of clarity, I address only issues related to cartilage or bone, although the same general experimental approach and logic can be used for other mesenchymal tissues.

cifically to form unique developmental structures or, in adults, to form repair blastemas, which are then capable of responding to local cues and differentiating accordingly to achieve regenerative repair (10,11).

Chick Limb Cells

More than 20 years ago, my collaborators and I attempted to define experimentally the conditions and cues necessary to control the differentiation of embryonic mesenchymal cells into cartilage and bone (5,7,17). Both in vivo and in vitro studies were used, but development of cell cultures and the general approach of using cell cultures has provided the experiential basis for approaching the study of mesenchymal stem cells from adults. The system we developed was the culturing of stage 24 (day 4.5) embryonic chick limb mesenchymal cells under conditions that promoted differentiation of cartilage (chondrocytes) (5,7,13,20) and bone (osteoblasts) (42,65).

Chondrocytes

Our first experimental effort with embryonic chick limb mesenchymal cells was to focus on chondrocyte development, which we learned was controlled by the initial plating density (5,17), oxygen levels (14), or, as recently shown by other investigators, a variety of physical and chemical factors (53,58,61). The key factor in the conversion of a mesenchymal cell to a chondrocyte is maintaining the progenitor cell in a round, unspread conformation. This can be accomplished simply by plating the cells initially under very compact, high-density conditions: 5×10^6 embryonic stage-24 limb mesenchymal cells per 35-mm dish (5,17). Even in a simple, defined medium consisting of insulin, transferrin, bovine serum albumin (BSA), and hydrocortisone in Eagle's minimum essential medium (MEM), the differentiation of chondrocytes and their further development can be documented as long as the cells are initially seeded at high density (18,30).

The high-density, limb cell-derived chondrocyte in culture makes two cartilage-specific molecules in abundance: type II collagen (68) and a large chondroitin sulfate, keratan sulfate proteoglycan (CSPG) (13,18,20). By detailed chemical and physical characterization of the CSPG synthesized on each day of culture, we showed that the glycosaminoglycan chains are biosynthesized slightly differently with

time (Fig. 2). Peptide maps show that the newly synthesized core protein (26) is identical on each day of culture, whereas the chondroitin sulfate chains are synthesized progressively shorter (30,000 D on day 2 to 15,000 D on day 20) and the keratan sulfate chains are synthesized progressively larger (0 to 10,000 D) (13,20). This biosynthetic progression is exactly what has subsequently been shown to occur in the cartilages of embryonic, adult, and aging human (50) and bovine specimens (62).

That embryonic chondrocytes have an aging-dependent program of changing biosynthesis is further documented when cultured embryonic chick chondrocytes are transplanted in a fibrin-based delivery vehicle into defects at the articular surface of adult chickens (29). Such chondrocytes produce what appears to be appropriate cartilaginous matrix and have been followed >18 months. The resulting repair cartilage appears to integrate perfectly into the defect and to provide the animal with a healthy, normal articular surface. These experiments and others clearly establish the concept of repairing cartilage with embryonic or appropriate reparative cells.

Osteoblasts

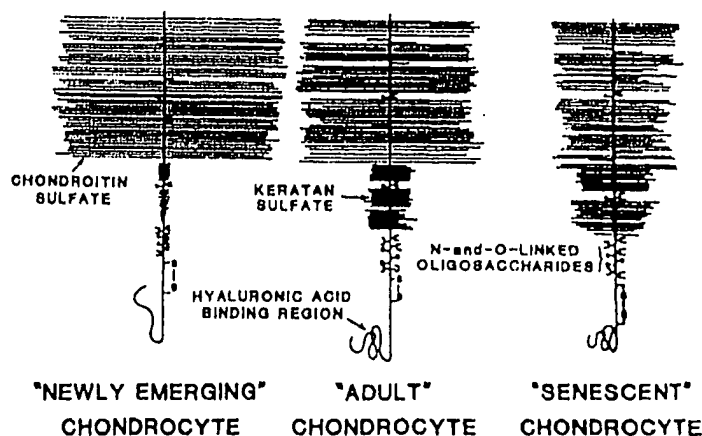
Our initial success in studying emergence of chondrocytes and formation of cartilaginous tissue from cultures of limb mesenchymal cells encouraged us to study differentiation of osteoblasts and formation of bone as well. Our initial logic was that high-density conditions caused cartilage formation and that cartilage was the progenitor tissue of bone. (Some investigators have reported that cartilage

provides the scaffold for bone formation.) After 2 years of frustrating experimentation, we realized that when infrequent bone and osteoblasts could be identified, the bone had formed at a distance from cartilage and never on or in the cartilage (42). By carefully decreasing the initial cell density of limb mesenchymal cells to just below the density at which some mineralized cartilage could form (2×10^6 cells/35-mm dish), we observed numerous deposits of bone and abundant osteoblasts which, again, were clearly at some distance from cartilage (6,42,44). In addition, these osteoblasts exhibited the classic response to parathyroid hormone (PTH) of elevated cyclic AMP levels (71,72) and possessed a bone-specific alkaline phosphatase (43). These studies clearly indicated that embryonic chick limb mesenchymal cells were capable of differentiating into osteoblasts and that the culture conditions supporting optimum osteoblast emergence were different from the conditions optimum for chondrogenesis.

Mouse and Human Limb Cells

With regard to cartilage and bone, the properties of mouse and human limb mesenchymal cells in culture appear to be quite similar, if not identical (25,46). Likewise, cartilage and bone development *in vivo* are also quite comparable, with the major exception that embryonic cartilage of chick does not calcify whereas that of mammals always calcifies (16). The comparable developmental properties of aves, rodents, and humans encourages us to continue experimentation with animal cells as an approximation of better understanding of the properties of human cells and tissues.

FIG. 2. Proteoglycans synthesized by newly differentiated, mature, and senescent chondrocytes. With increasing age, chondrocytes synthesize proteoglycans that have smaller chondroitin sulfate chains and larger keratan sulfate chains (7,8,12,13,20).



LINEAGE OF MESENCHYMAL CELLS

Cartilage

The important inference from the above discussion is that chondrocytes have a programmed (i.e., genetically dictated) sequence of changes in their end-stage expression (8,12). The differences in glycosaminoglycan chain lengths or chemistry are stable to cell culturing or metabolic perturbation. The control of these events is not known, but all experiments designed to slow this sequence of biosynthetic alterations or reverse them have failed. The inference is that a genomic mechanism somehow "tells time" and that this clock is hard-wired and unidirectional (8,12).

Such biosynthetic changes in articular cartilage are different from the lineage changes observed in adult growth plate or embryonic limb cartilage. A discrete set of expressional stages or lineage states, comprising dividing, maturing, and hypertrophic chondrocytes, is apparent in embryonic limb tissue, cell culture (13,58,61), and in the growth plate (19,28). Eventually, the hypertrophic cartilage in vivo is eroded by vascular, marrow, and phagocytic cells and replaced by bone. Each chondrocytic lineage state is uniquely different from its predecessor, as shown in Fig. 3. For example, hypertrophic chondrocytes synthesize a unique small collagen, type X, and a unique proteoglycan (54,55); neither of these molecules is synthesized by mature chon-

drocytes. In this particular circumstance, several factors are proposed to contribute to conversion of mature chondrocytes to hypertrophic chondrocytes (35); reversal of this process has not been reported.

Bone

We recently reviewed the major aspects of embryonic bone development. Figure 4 shows several important elements or rules governing this complex process (10,11,16). First, a discrete positioning of progenitor cells, stacked cells, existed in proximity to the developing bone (47). The stacked cells give rise to osteoblasts in a discrete series of lineage steps (described below). The end stage or secretory osteoblast is positioned by its proximity to vasculature, with the "back" of the osteoblast to the capillary and osteoid deposited from the "front" of this highly oriented secretory cell (47,48). The vasculature is the orientor of osteogenesis and the osteoblast is the formative element. Cartilage is not replaced by bone, but is instead the target for vascular (marrow) replacement (48); in the early limb, the cartilage model exactly defines the eventual marrow cavity.

That a discrete series of individual lineage stages exists between the progenitor cells in the stacked cell layer and the secretory osteoblasts is now clear, as shown in Fig. 5. We recently isolated four monoclonal antibodies, SB1, 2, 3, and 5, which have helped provide evidence for an osteoblast lineage (3,4). Progenitor cells in the stacked cell layer and osteocytes do not interact with SB1, 2, or 3. Newly differentiated osteogenic cells react with SB1, but not with SB2 or 3, whereas fully secretory osteoblasts react with SB1, 2, and 3. A subpopulation of osteogenic cells reacts with SB2, but not SB3. Osteocytes react with OB7.3 of Nijweide and Mulder (38) or with our SB5, but not with SB1, 2, or 3. The lineage tree in Fig. 3 is based on these observations and not only establishes the existence of an osteoblastic lineage but suggests that osteocytes are derived directly from osteoblasts with SB1, 2, and 3 antigens that are suppressed as SB5 and OB7.3 are turned on. Experiments are now in progress to use these monoclonal antibodies to isolate representatives of each lineage stage so that studies can be conducted to identify the agents that promote the progression from one lineage stage to the next. Central to the thesis presented below is the existence of osteoprogenitor cells in the stacked cell layer, the future periosteum.

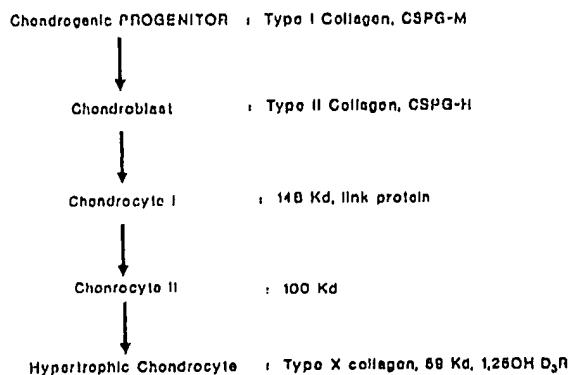
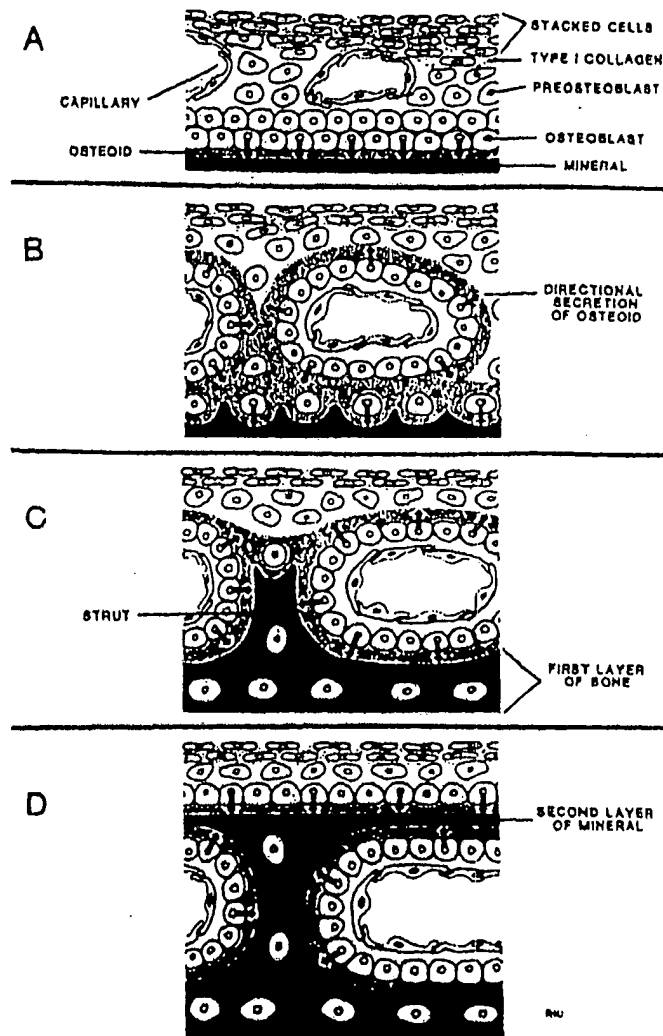


FIG. 3. Chondrogenic lineage. Based on the experiments of Solorush et al. (58,61) a hypothetical lineage map can be constructed to consist of at least five separate stages based on the changing biosynthesis of proteins (named or by molecular weight, K_d) or chondroitin sulfate proteoglycan (CSPG). The receptor for 1,25-dihydroxy Vitamin D_3 is represented as 1,25OHD $_3$ R.

FIG. 4. Sequence of progressive in vivo bone development. Progressive repositioning of the vasculature from outside the stacked cell layer to a position in close approximation to the first layer of secretory osteoblasts responsible for formation of the first bony collar of the chick tibia (11,47,48). The osteoblast is oriented with its back toward the invading capillary and secretion of osteoid toward the cartilage core from the osteoblast's face. In this model, osteoblasts secrete osteoid in a direction away from vasculature (B), causing formation of a strut (C) and eventually forming the second layer of bone (D). These observations show that an intimate relationship exists between vasculature and newly forming bone.



BIOACTIVE FACTORS IN BONE

From the earliest days of modern humans, bone has been recognized to have the powerful capacity to repair discontinuities (22). A variety of bioactive factors combine in a complex multicellular, multi-step response in which reparative cells are specifically attracted to the repair site. These cells then aggregate, multiply, bridge the bone gap, and differentiate into chondrocytes or osteoblasts as controlled by the proximity to vasculature. Recently, an intensive research activity to identify and characterize these various bioactive factors was largely

successful (56,66,67,69). Our laboratory has described the purification of a protein factor, chondrogenic stimulating activity (CSA), which converts embryonic limb mesenchymal cells to chondrocytes (63,64). We are also attempting to purify a bone-derived chemoattractant for mesenchymal cells by using the now standard modified Boyden chamber (31,33).

Relevant to the thesis developed below, the identity and manipulation of the cells responding to bone-derived bioactive factors is directly related to successful bone repair. Such responding cells are present in the adult periosteum (36), dermis (49),

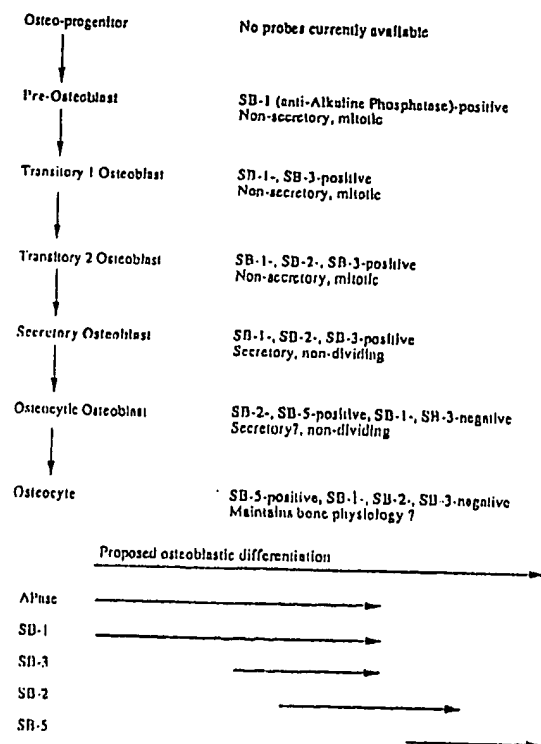


FIG. 5. Osteogenic cell lineage. Based on recent experimentation in which monoclonal antibodies were generated to cell surface antigens of osteogenic cells (3,4), a tentative lineage diagram reflects acquisition or loss of specific antigenic determinants. The characteristics of SB1, 2, and 3 were described previously (3); SB-5 (4) has been characterized and is similar to OB7.3 of Nijweide and Mulder (38). The individual lineage states are not weighted in terms of their prevalence or dwell-time; e.g., "transitory osteoblast 1" occurs rarely and cannot be recognized easily except at specific times and locations, whereas the "secretory osteoblast" is easily recognized and plentiful.

bone marrow (1,40,41,45), and connective tissue associated with muscle (34,37). One or all of these repositories are capable of forming bone when appropriately delivered bioactive factors are presented.

Alternately, when the responsive cells, stem cells, are placed in suitable delivery vehicles that can retain these cells while encouraging vascular invasion, bone can be observed to form. Recently, we used calcium phosphate porous ceramics in composite with marrow to encourage bone formation at both heterotopic and orthotopic sites (40,41). Whole disaggregated marrow cells in suspension are loaded into porous ceramic and transplanted to subcutaneous, intramuscular, or bone defect sites

in vivo. In 1-2 months, the few mesenchymal stem cells in the marrow have replicated massively and differentiated into osteoblasts. In the dead-end pores of the ceramic, which are devoid of vasculature, these stem cells differentiate into chondrocytes and form cartilage.

MESENCHYMAL STEM CELLS

From the above discussion several key facts are evident. First, embryonic mesenchymal stem cells in the limb which give rise to cartilage and bone in vivo can be manipulated in vitro. Second, these cells have a lineage progression of separate, individual steps, whether it be the chondrogenic or osteogenic pathway. Third, local cuing, sometimes involving highly potent protein factors, is responsible for providing positional information and causing lineage progression. Cell culture conditions have been refined to the extent that not only can these progressive events be studied in detail, but manipulation of the cells is also possible to provide control of tissue size and function.

Fourth, although chondrocytes and osteoblasts are derived from a common mesenchymal cell, the conditions for their initial differentiation and progression through the individual steps of their lineages are uniquely different. For example, osteogenesis is dependent on proximity to vasculature whereas chondrogenesis requires the complete absence of vasculature (7,10,11,16); osteogenesis is optimum at an initial cell culture seeding density in 35-mm dishes of 2×10^6 embryonic limb mesenchymal cells, whereas chondrogenesis is optimum at 5×10^6 cells (5,17,42).

Fifth, bone forms from mesenchymal stem cells in a cartilage-independent manner with vasculature providing a determinative discriminator between these two tissues; embryonic cartilage is not replaced by bone, but rather by vasculature and marrow (10,11,16). Sixth, we can demonstrate that three tissue sites are the repositories of mesenchymal stem cells: marrow (1,40,41,45), periosteum (36), and muscle connective tissue (34,37).

MARROW

Figure 6 outlines an assay to demonstrate that marrow contains mesenchymal stem cells capable of differentiation into cartilage and bone. Whole marrow is disrupted into single cells by passing it through needles of successively smaller sizes; the

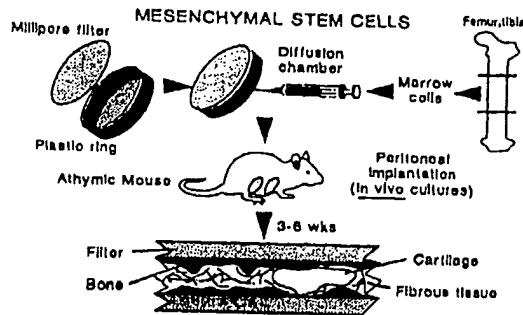


FIG. 6. Diffusion chamber assay in nude mice. Cell samples from marrow or other sources can be loaded into chambers composed of two Millipore filters glued to the edges of a plastic ring. These chambers are then implanted in the peritoneal cavity of athymic (nude) mice as a highly vascular in vivo incubation site. The filters prevent host cells from entering the chambers but permit rapid diffusion of nutrients and other factors into or out of the chamber. Histologic identification of two distinctive phenotypes, cartilage and bone, indicates that mesenchymal stem cells were present in the initial inoculum (1,2,45).

cells are counted, and $1-10 \times 10^6$ cells are placed in a small diffusion chamber (1,2,45). This chamber is of simple construction consisting of a small plastic ring onto which two Millipore filters have been glued. The filters allow body fluids (salts, nutrients, proteins, large protein complexes) to pass in and out of the chamber, but cells inside are not mixed with host cells, and tissues such as the vasculature are completely excluded. These chambers are implanted into the peritoneal cavity of an athymic (nude) mouse as an in vivo incubator, and they quickly become surrounded by host vasculature. Detailed studies have shown that the hematopoietic cells are eliminated, whereas mesenchymal cells vigorously divide and differentiate into cartilage in the middle of the chamber and bone at the filter interfaces closest to the enveloping vasculature (1,2,45). The presence of both cartilage and bone in the diffusion chamber has been compared to the presence of predominantly bone inside the highly vascularized pore regions of porous calcium phosphate ceramics loaded with marrow cells and implanted at heterotopic or orthotopic sites described above (40,41).

As a refinement of these experiments, we have been able to purify marrow mesenchymal cells by their differential adhesion to culture dishes and have successfully cultured cells through many passages (23). These cultured marrow mesenchymal cells from rat or chicken retain their capacity to differentiate into osteoblasts in ceramics through

such subculturing. Of importance is the demonstrated success of isolating marrow mesenchymal cells and mitotically expanding these cells with retention of their full developmental potency to differentiate into osteoblasts or chondrocytes.

Periosteum

Another repository for mesenchymal stem cells is the periosteum, a complex layer of cells that composes the outermost layer of long bone; we have termed the periosteum the stacked cell layer in developing embryos (1,16,47,48). This layer clearly responds to injury by rapidly expanding and forming woven bone; it also has cells capable of differentiating into chondrocytes when the periosteum is transplanted into an articular cartilage defect (39). In experimentation paralleling that described above for marrow mesenchymal cells, we have been successful in culturing and passaging periosteal cells (36). In porous ceramics implanted in nude mice, these cultured periosteal cells differentiate into osteoblasts (36). When the same cell preparation is injected into a subcutaneous site in a nude mouse, the cultured periosteal cells differentiate into both bone and cartilage (36). The important point is that culture-expanded periosteal cells retain their full developmental potency and can be manipulated to form two very complex and different tissues, bone or cartilage.

THE FUTURE: (SELF-CELL THERAPY)

Several important conceptual and technical advances have converged to allow us to consider the possibility of using a patient's own mesenchymal stem cells as starting material for tissue repair protocols. Mesenchymal stem cells must exist to maintain the living organisms, just as hematopoietic stem cells must exist to support both red and white blood cell turnover. Developmental biology has taught us that differentiated cells arise in a sequence of definitive cellular and molecular transitions, a lineage, from stem cell to end phenotype. Bone, for example, turns over; new osteoblasts arise, have a defined half-life, make new bone, and then die, to be replaced by other newly differentiating end-stage osteoblasts. Such osteoblasts must arise from stem cells; thus, a living organism must have repositories of stem cells.

Therefore, we might be able to isolate such human mesenchymal stem cells and place them in cell

culture, where we could mitotically expand their numbers. Eventually, if we had enough of these cells, we could reintroduce them into the original donor in a manner that guaranteed that they would massively differentiate into a specific tissue, such as cartilage or bone, at a transplantation or repair site. Immunorejection would not be a problem because the donor and host would be one and the same.

The first experimental step to test this idea is to determine if the animal-based technology described above can be modified to be used with human material. The first attempts at this have been highly encouraging. Recently, human marrow was introduced into diffusion chambers which were placed in nude mice; both cartilage and bone were eventually observed in the chamber (2). We recently cultured human marrow and isolated mesenchymal cells that were passaged, introduced into porous ceramics, and implanted subcutaneously in nude mice. In the pore regions of these highly vascularized composites, bone clearly formed in every sample of culture-expanded, marrow-derived mesenchymal cells tested (27). These preliminary experiments provide hope that the animal-based technology developed for mesenchymal cells from marrow or periosteum will be translatable to humans.

The concept of ex vivo manipulation of cells and their reimplantation into a donor is the basis for proposing self-cell therapy as a future possibility. Massive bone regeneration to fill gaps from tumor excision, regeneration of damaged articular cartilage, and maintenance of bone formation in the elderly at risk for osteoporosis are clinical protocols that require large numbers of the appropriate reparative skeletal cells. The patient's own mesenchymal stem cells may prove to be the basis of a new, cell-based treatment plan requiring the merging of molecular biology to produce specific bioactive factors, cell biology to develop ex vivo manipulation regimens, and surgeons able to implant cells capable of repairing skeletal defects by the regeneration process.

Acknowledgment: I thank the members of my laboratory, both past and present, for providing the fabric and labor of the cloth of our scientific pursuits. The resultant material of many colors provides both the backdrop and carpet for our scientific accomplishments and progress. My thanks are not enough to repay their kindnesses, contributions, and stimulation. This work was supported by grants from the NIH.

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EVIDENCE APPENDIX

ITEM NO. 20

Declaration of Dr. Richard Heuser filed on June 17, 2003

CERTIFICATE OF MAILING

03 JUN 17 12:13:51

I hereby certify that the attached DECLARATION OF RICHARD HEUSER, M.D. was delivered to the Assistant Commissioner for Patents by the undersigned from Arrow Intellectual Property Services, 2001, Jefferson Davis Highway, Suite 602, Arlington, Virginia 22202, by hand carrying said DECLARATION to Art Unit 1646, Crystal Plaza 1, Tenth Floor, Attention: Examiner Elizabeth C. Kemmerer this 17th day of June, 2003.

Dated: June 17, 2003

Ann Rutledge
Printed Name: Ann Rutledge

Docket No. ~~XXXXXX~~: 1000-10-CO1
Serial No. ~~XXXXXX~~: 09/836,750
Filed/Registered ~~XXXXXX~~: 04/17/01
Due Date: - - -

ARROW INTELLECTUAL PROPERTY SERVICE

The Patent Office acknowledges, and has stamped hereon, the date of receipt of the items check below:

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- ☐ Amendment/Response
- ☒ ~~XXXXXX~~ Declaration of ~~XXXXXX~~ of Richard Heuser
- ☐ Brief/Reply Brief/Notice of Appeal
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COPY

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
SERIAL NO.: 09/836,750)	
FILED: April 17, 2001)	EXAMINER: E.C. Kemmerer, Ph.D.
FOR: METHOD AND APPARATUS)	
FOR INSTALLATION OF)	GROUP ART UNIT: 1646
DENTAL IMPLANT)	

DECLARATION OF RICHARD HEUSER, M.D.

I, Richard Heuser, declare as follows:

1. I have offices at 525 North 18th Street, Suite 504, Phoenix, Arizona 85006.
2. My Curriculum Vitae is attached hereto as Exhibit A.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. A copy of such disclosures is attached hereto as Exhibit B.
4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method of using a growth factor for growing muscle in a human heart.

5. I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit C along with a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary. A service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe that both definitions are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.
6. I have read and understood the claims set forth in Exhibit D and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.
7. The materials included in attached Exhibit E illustrate that placement of a growth factor in a human patient causes muscle growth in a heart. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.
8. Based upon above Paragraphs 3-7, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.

9. Based upon above Paragraphs 3-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit D without need for resorting to undue experimentation.
10. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date:

6/5/03

Richard Heuser
Richard Heuser

EXHIBIT A

CURRICULUM VITAE

Curriculum Vitae
Richard Ross Heuser, M.D., F.A.C.C., F.A.C.P.

ADDRESS:

525 North 18th Street, Suite 504
Phoenix, Arizona 85006
(602) 234-0004
(602) 234-0058 (fax)
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EDUCATION:

1969 - 1972	University of Wisconsin Honors in Chemistry Phi Beta Kappa Evan Helfaer Scholarship in Chemistry
1972 - 1976	University of Wisconsin School of Medicine Graduation with Honors - May 1976 Alpha Omega Alpha Evan Helfaer Scholarship in Medicine

POST GRADUATE TRAINING:

1976 - 1977	Internship in Medicine The Johns Hopkins Hospital Baltimore, Maryland
1977 - 1979	Residency in Medicine The Johns Hopkins Hospital Baltimore, Maryland
1979 - 1981	Fellowship in Cardiology The Johns Hopkins Hospital Baltimore, Maryland

LICENSURE:

State of Arizona, License #19703
State of New Mexico, License #83-220

EMPLOYMENT:

December 2002 - Present	Director of Cardiovascular Research St. Joseph's Hospital and Medical Center Phoenix, Arizona
April 2001 - Present	Cardiac Cath Lab Director St. Luke's Medical Center, Phoenix, Arizona
June 2000 - Present	Medical Director Discovery Alliance, Phoenix, Arizona
1998 - June 2000	Director Phoenix Research Center, Phoenix, Arizona

April 1997 - Present	Medical Director Phoenix Heart Center, Phoenix, Arizona
December 1999 - Present	Director of Research St. Luke's Medical Center, Phoenix, Arizona
April 1997 - December 1999	Director of Research and Education Phoenix Regional Medical Center, Phoenix, Arizona
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute, Phoenix, Arizona
July 1983 - April 1990	Private Practice New Mexico Heart Clinic, Albuquerque, New Mexico
July 1982 - June 1983	Private Practice Houston Cardiovascular Associates, Houston, Texas
June 1981 - July 1982	Instructor in Medicine, Cardiology The Johns Hopkins Hospital, Baltimore, Maryland

PROFESSIONAL APPOINTMENTS:

1981 - July 1982	Instructor in Medicine - Cardiology Division of Cardiology The Johns Hopkins Hospital, Baltimore, Maryland
July 1982 - June 1983	Instructor in Medicine, Cardiology Baylor College of Medicine, Houston, Texas
July 1983 - February 1990	Director, Interventional Cardiology New Mexico Heart Clinic, Albuquerque, New Mexico
April 1984 - June 1986	Clinical Assistant Professor of Medicine University of New Mexico, Albuquerque, New Mexico Director, Medical Residency Program New Mexico Heart Clinic, Albuquerque, New Mexico
June 1986 - April 1990	Clinical Associate Professor of Medicine University of New Mexico, Albuquerque, New Mexico
May 1996 - April 1997	Director, Interventional Cardiology Arizona Heart Institute Foundation, Phoenix, Arizona
Sept 1995 - December 1999	Medical Director - Cardiac Catheterization Laboratory Phoenix Regional Medical Center, Phoenix, Arizona
December 1990 - Present	Clinical Associate Professor of Medicine University of Louisville, Louisville, Kentucky
April 1990 - April 1997	Director of Research and Education Arizona Heart Institute Foundation, Phoenix, Arizona

April 1997 - December 1999 Director of Research and Education
Phoenix Regional Medical Center, Phoenix, Arizona

BOARD MEMBERSHIPS:

American Board of Internal Medicine
American Board of Cardiovascular Diseases, Diplomat
American Board of Interventional Cardiovascular Diseases, Diplomat

PROFESSIONAL MEMBERSHIPS:

Fellow, American College of Angiology
Fellow, American College of Cardiology
Fellow, American College of Physicians
Fellow, of the American Heart Association
Fellow, American Society of Cardiovascular Interventions
Fellow, International Society of Cardiovascular Interventions
Fellow, Society for Cardiac Angiography and Interventions
Member, American Association for the Advancement of Science
Member, American Heart Association
Member, American Medical Association
Member, Houston Cardiology Society
Member, Houston Society of Internal Medicine
Member, International Andreas Grüntzig Society
Member, International Network of Interventional Cardiology
Member, International Society for Carotid Artery Therapy
Member, International Society for Minimally Invasive Cardiac Surgery
Member, New Mexico Medical Society
Member, Harris County Medical Society
Member, Texas Medical Association
Member, National Register's Who's Who in Executives and Professionals
Member, Who's Who in Medicine and Healthcare 2002-2003

CLINICAL ADVISORY BOARDS:

Advanced Cardiovascular Systems
USCI
Mansfield Scientific Interventional Board
Medtronic Interventional Vascular
Scientific Advisory Board of International Society of Heart Failure

EDITORIAL BOARDS:

Catheterization and Cardiovascular Diagnosis
Journal of Endovascular Surgery
Cardiovascular Research Foundation/Society of Cardiac Angiography and Interventions
Abstract Grader TCT

DATA SAFETY BOARDS:

- ICEM Data Safety Monitoring Board

Abbott Laboratories Data Safety Monitoring Board for Drug Coated Stent Program, PREFER, A Perspective STUDY to Evaluate the Safety and Efficacy of the ABT-578 coated BiodivYsio® Stent for the Reduction of Restenosis

CONSULTANT TO:

Editors of the *Annals of Internal Medicine*
 Editors of *Catheterization and Cardiovascular Diagnosis*
 Editors of *Circulation*
 Editors of the *Journal of Invasive Cardiology*
 Editors of the *American Journal of Cardiology*
 Editors of *Web M.D.*
 Annual Scientific Session Program Committee of the American College of Cardiology
 Annual Scientific Session Program Committee of the American College of Cardiology
 Abstract Advisor for Angioplasty; Stents
 Annual International Symposium of Transcatheter Cardiovascular Therapeutics
 Abstract Grader

DEVICE RESEARCH:

Sub-Investigator	ACS Multi-Link Stent Trial Principal Investigator - ACS RX
Principal Investigator	ACT-One Trial Principal Investigator - Angio-Seal Trial
Principal Investigator	Balloon Expandable Intraluminal Stent for Subtotally Occluded Iliac Arteries
Principal Investigator	Bard® Memotherm Carotid Stent Study
Principal Investigator	BARRICADE Trial - The Barrier Approach to Restenosis: Restrict Intima and Curtail Adverse Events (JOMED JOSTENT)
Principal Investigator	BEST Trial
Principal Investigator	BetaCath System Trial
Principal Investigator	Boehringer Ingelheim Pharmaceuticals Protocol Comparing Micardis and COZAAR
Principal Investigator	CABERNET Clinical Trial - Carotid Artery Revascularization using the Boston Scientific EPI FiltreWire EX™ and the EndoTex™ NexStent™
Principal Investigator	CADILLAC Trial
Principal Investigator	CAPRICORN Trial
Principal Investigator	CAPTIVE - Cardioshield Application Protects During Transluminal Intervention of Vein Grafts by Reducing Emboli
Principal Investigator	CARDIOMETRICS
Principal Investigator	Carotid Wallstent Trial
Principal Investigator	CAVEAT II Trial
Principal Investigator	Clinical Investigation of the Magnum Wire vs. Standard Guide Wires during Total Occlusion Angioplasty
Principal Investigator	Cook GR II Trial
Principal Investigator	CORDIS Nitinol Carotid Stent And Delivery System for the Treatment of Obstructive Carotid Artery Disease
Principal Investigator	Cordis Carotid Randomized Sapphire
Principal Investigator	Cordis Bilateral AAA Device & Delivery System
Principal Investigator	(CATS) Safe-Steer™ Wire System Coronary Artery Total Occlusion Study
Principal Investigator	CREDO Trial
Principal Investigator	Novoste CUP Trial
Principal Investigator	CVD Accucath Infusion Catheter
Principal Investigator	Duett Closure Device
Principal Investigator	EndoSonics Cath scanner Oracle - PTCA Catheter

Principal Investigator EPI FilterWire-EX™ System During Transluminal Intervention of Saphenous Vein Grafts

Principal Investigator Extra Stent

Principal Investigator GREAT - Guided Radio Frequency Energy Ablation of Total Occlusions Using the Safe Cross™ Radio Frequency Total Occlusion Crossing System

Principal Investigator GRIP - Guided Radio Frequency in Peripheral Total Occlusions using the Safe-Cross™ Radio Frequency (RF) Total Occlusion (TO) Crossing System

Principal Investigator HIPS Trial

Principal Investigator Human Percutaneous Laser Angioplasty of the Coronary Arteries

Principal Investigator Johnson & Johnson Intracoronary Stent Program Supplement #27 "New" Delivery System

Principal Investigator Kensey Nash Hemostatic Puncture Closure Device

Principal Investigator Mansfield-Boston Scientific Strecker Coronary Stent

Principal Investigator Medtronic AVE S7 with Discrete Technology Coronary Stent System

Principal Investigator Medtronic AVE S7 Coronary Stent Registry

Principal Investigator MOBILE Trial - More Patency with Beta for In-Stent Restenosis in the Lower Extremities Trial IDE #G010295; Protocol D00789 Rev B dated 12/01

Principal Investigator NIR Stent Trial

Principal Investigator Neurex/Elan Pharmaceuticals Trial

Principal Investigator PAMI Stent Trial

Principal Investigator Paragon Stent

Principal Investigator Paris Radiation Trial

Principal Investigator PaS Trial

Principal Investigator Percutaneous Coronary Angioscopy in Unstable Angina

Principal Investigator Percutaneous Recanalization of Stenotic Human Coronary Arteries with Balloon Expandable Intracoronary Stents

Principal Investigator Percutaneous Recanalization of Stenotic Human Saphenous Vein Bypass Graft with Balloon Expandable Intraluminal Stents

Principal Investigator Percutaneous Thermal Balloon Angioplasty

Principal Investigator PMR Trial

Principal Investigator Pravastatin or Atorvastatin Evaluation and Infection Therapy (Prove It)

Principal Investigator Presto Trial

Principal Investigator RAVES Trial

Principal Investigator RESCUE Trial

Principal Investigator SAFER - Saphenous Vein Graft Angioplasty Free of Emboli Randomized Study Using the PercuSurge Guard Wire™ System

Principal Investigator SAVED Trial

Principal Investigator Schering-Plough Phase III Study of SCH 58235 in addition to Pravastatin compared to placebo in subjects with primary hypercholesterolemia

Principal Investigator Long-Term, Open-Label, Safety and Tolerability Study of SCH 58235 in Addition to Pravastatin in Patients with Primary Hypercholesterolemia

Principal Investigator Schneider WINS Trial

Principal Investigator SCORES Trial

Principal Investigator Sepracor Study of Norastemizole in Cardiac Compromised Subjects

Principal Investigator **SMART Trial (National PI)**

Principal Investigator SMART: Post-Approval Study

Principal Investigator SNAPIST - A Phase 2, Safety Study of Systemic Nanoparticle Paclitaxel (ABI-007) For In-Stent Restenosis; IND #63,082

Principal Investigator SOAR - Renal Stent

Principal Investigator Efficacy and Safety Study of the Oral Direct Thrombin Inhibitor H 376/95 Compared with Dose-Adjusted Warfarin (Coumadin) in the Prevention of Stroke and Systemic Embolic Events in Patients with Atrial Fibrillation (SPORTIF V)

Principal Investigator STARS Trial

Principal Investigator **START Trial (National PI)**

Principal Investigator STRATUS Trial

Principal Investigator STRESS III Trial

Principal Investigator	SUMO Trial
Principal Investigator	(SWING) Sound Wave Inhibition of Neointimal Growth
Principal Investigator	Talent Endoluminal Graft (High Risk & Low Risk)
Principal Investigator	Talent Endoluminal Spring Stent-Graft System
Principal Investigator	Tenax-XR Coronary Stent System
Principal Investigator	TITAN Trial
Principal Investigator	Trimedynne Excimer Laser Assisted Percutaneous Coronary Angioplasty
Sub-Investigator	Trimedynne Percutaneous Eclipse Holmium Laser Coronary Angioplasty
Principal Investigator	VeGAS 2 Trial
Principal Investigator	Velocity Trial Principal Investigator - Venus Stent
Co-Investigator	WALLSTENT Study
Principal Investigator	WIKTOR Coronary Stent

PHARMACOLOGY RESEARCH:

Principal Investigator	Abbott rUK Trial
Principal Investigator	Ajinimoto Pharmaceuticals Double-Blind Placebo-Controlled Study of AT-1015 in Patients with Intermittent Claudication due to peripheral arterial disease
Sub-Investigator	Amgen, Inc. Anakinra Trial for Rheumatoid Arthritis
Principal Investigator	Astra Zeneca Pharmaceutical Trial to Evaluate the Safety and Efficacy of XXXX and Atorvastatin
Principal Investigator	Astra Zeneca Trial Open Label Dose Comparison Study to Evaluate the Safety and Efficacy of Rosuvastatin versus Atorvastatin, Pravastatin, and Simvastatin in Subjects with Hypercholesterolemia
Principal Investigator	Parke-Davis and Pfizer Randomized Open-Label Study Comparing the Efficacy of Once Daily Atorvastatin to Simvastatin in Hypercholesterolemic Patients
Principal Investigator	Pilot Study to Evaluate Intracoronary Administration of Activase for the Treatment of Intracoronary Thrombus
Principal Investigator	Artistic Trial
Principal Investigator	AstraZeneca Trial of Niaspan versus New Generation Statin for the Treatment of Type IIB and Type IV Hyperlipidemia
Principal Investigator	AstraZeneca Multicenter Trial for drug (XXX) and Atorvastatin for the Treatment of Hypercholesterolemia
Principal Investigator	BRAVO Trial
Principal Investigator	BioVail Angina & Hypertension Trial
Principal Investigator	CAPRICORN Trial
Principal Investigator	Challenge Trial
Sub-Investigator	Comparison of Lopentol and Omnipaque in Adult Angiocardiology
Sub-Investigator	Comparison of Intravenous Adenosine to Intravenous Placebo in Termination of Spontaneous or Induced Paroxysmal Supraventricular Tachycardia
Principal Investigator	Centocor Chimeric 7E3 Fab
Principal Investigator	COR Therapeutics Randomized Placebo-Controlled Dose Ranging Study of drug (XXXX) in Patients with Atherosclerotic Cardiovascular, Peripheral Vascular, and/or Cerebrovascular Disease
Sub-Investigator	Dose Response Study of Bucindolol in Patients with Congestive Heart Failure
Principal Investigator	Effects of Recombinant Human Superoxide Dismutase in Patients with Acute Myocardial Infarction Subject to Coronary Artery Reperfusion
Sub-Investigator	Eli Lilly - Agitation/Alzheimer's Trial
Principal Investigator	EPILOG Trial
Principal Investigator	ERASER Trial
Principal Investigator	GUSTO Trial
Principal Investigator	A multi-center, randomized, double blind, placebo-and-active controlled Parallel Group Dose-ranging Study of the HMG CoA Reductase Inhibitor, BMS-423526, in the treatment of Hyperlipidemia

Principal Investigator	Study Lovastatin XL with MEVACOR in patients with hypercholesterolemia
Sub-Investigator	Lovastatin Multi-Center Trial
Principal Investigator	Extended Trial of Lovastatin XL for the treatment of hypercholesterolemia
Principal Investigator	Multicenter Double-Blind Placebo controlled trial of drug (XXXX) in patients with Type 2 Diabetes and Congestive Heart Failure
Principal Investigator	Effect of LDL-Cholesterol Lowering Beyond Currently Recommended Minimum Targets on coronary heart disease (CHD) Recurrence in patients with Pre-Existing CHD
Principal Investigator	A Double-Blind, Multi-Center, Randomized, Placebo-Controlled, Parallel Group Dosing Study Evaluating the Effects of Nebivolol on Blood Pressure in Patients with Mild to moderate Hypertension, NEB 302
Principal Investigator	Parallel Group Extension Study to Determine the Safety and Efficacy of Long-Term Nebivolol Exposure in Patients with Mild to Moderate Hypertension NEB 306,
Sub-Investigator	NeoTherapeutics Alzheimer's Disease 2000
Sub-Investigator	NeoTherapeutics Alzheimer's Disease 2001
Principal Investigator	OCTAVE Trial
Sub-Investigator	OCTAVE Trial
Principal Investigator	Pfizer Phase II Multicenter, double-blind placebo controlled randomized parallel group dose ranging study of the safety of CP529,414 soft-gel capsules
Principal Investigator	PLAC Trial
Principal Investigator	Protocol 073 Trial
Principal Investigator	Knoll Pharmaceutical Double-Blind Randomized Clinical Trial of Slow Release Propafenone (Rythmol-SR®) in the Prevention of Symptomatic Recurrences of Atrial Fibrillation
Principal Investigator	PREVAIL - A Phase 2 Multicenter, Double-Blind Placebo-Controlled, Dose-Ranging Study to Evaluate the Safety and Efficacy of BO-653 in Prevention of Post-Angioplasty Restenosis in Stented Lesions
Principal Investigator	PROVE-IT TIMI 22 - Pravastatin or Atorvastatin Evaluation and Infection Therapy
Principal Investigator	PURSUIT Trial
Principal Investigator	QUIET Trial
Principal Investigator	RAFT Trial
Principal Investigator	REPLACE Randomized Evaluation in PCI Linking Angiomax to reduce Clinical Events
Sub-Investigator	Safety and Efficacy Study of Burroughs - Wellcome Tissue Plasminogen Activator in Patients with Acute Myocardial Infarction
Principal Investigator	A 6-week, open-label, dose-comparison study to evaluate the safety and Efficacy of Rosuvastatin versus Atorvastatin, Cerivastatin, pravastatin, and Simvastatin in subjects with hypercholesterolemia
Principal Investigator	A 48-week, open-label, non-comparative, Multicentre, Phase IIIb study to evaluate the efficacy and safety of the Lipid-Regulating agent Rosuvastatin in the treatment of subjects with Fredrickson Type IIa and Type IIb Dyslipidemia, including Heterozygous Familial Hypercholesterolemia
Principal Investigator	SAGE Trial
Sub Investigator	Long Term Open Label Safety and Tolerability Study of SCH58235 in addition to Pravastatin in Patient With Primary Hypercholesterolemia
Principal Investigator	Phase III Double-Blind Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
Principal Investigator	Phase III Open Label Efficacy and Safety Study SCH58235 (10 mg) in Addition to Pravastatin Compared to Placebo in Subjects with Primary Hypercholesterolemia
Principal Investigator	Sepracor Protocol Study of Norastemizole in Cardiac Compromised Subjects
Principal Investigator	SPORTIF V - Atrial Fibrillation Trial
Principal Investigator	SWORD Trial
Principal Investigator	Titration-to-Response Trial Comparing Micardis and COZAAR® in Patients with Mild-to-moderate Hypertension

Principal Investigator TNT Trial
 Principal Investigator TREND Trial
 Sub-Investigator VALDECOXIB Trial
 Principal Investigator An Open-Label, Multinational, Multicentre, Extension Trial to Assess the
 Long-Term Safety and Efficacy of ZD4522 in Subjects in the ZD4522 Clinical Trial Program

BASIC RESEARCH:

- 1990 - 1993 Systematic assessment of Medtronic balloons and guiding catheters in porcine and canine models. Sponsored by Medtronic, Inc.
- 1990 - 1993 Determination of radiopacity and torquability of Medtronic vascular catheters in porcine models. Sponsored by Medtronic, Inc.
- 1992 - 1996 Evaluation of Strecker stent in porcine and canine models.
Sponsored by Boston Scientific
- Evaluation of Wiktor stent and stent in porcine and canine models.
Sponsored by Medtronic, Inc.
- Evaluation of NIR stent in porcine models.
Sponsored by Cordis Corp.
- 1990 - 1994 Evaluation of Japan Crescent radiofrequency balloon in porcine model with emphasis on histopathology of heat-produced lesions. Abstract submitted at 1993 AHA Conference.
- 1993 Evaluation of radiofrequency wire for total coronary occlusions in porcine models: Determining energy limitations. Equipment subsequently licensed to Radius Medical.
- 1994 - 1997 Training courses for professionals (physicians, engineers, technicians) in techniques and strategies for placement of coronary stents. Five courses sponsored by Johnson & Johnson, Medtronic, Inc. and Cook, Inc.
- 1997 Efficacy of the Endotex Abdominal Aortic Aneurysm exclusion device in a porcine model gauging ability to exclude renal arteries, ease of placement and radiopacity. Sponsored by Endotex
- 1998 Use of percutaneous myocardial revascularization in a porcine model.
Sponsored by Cardiogenesis Corporation at Stanford University.
- 1998 - 1999 Utility of radiofrequency (RF) percutaneous myocardial revascularization in acute and chronic porcine model: Histopathology and angiogenesis related to use of RF alone and in combination with growth factor (VEGF). Results presented at Angiogenesis 1999, Washington, DC.
- 1999 Development and testing of embolic probe device in porcine model (patent pending). Performed at PRMC and separately at Columbia Presbyterian in New York.
- 1999 Evaluation of the Medtronic carotid and SVG stent in porcine carotid and saphenous vein graft lesions assessing ease of use and 30-day outcome.
Sponsored by Medtronic, Inc.
- 1999 Development and testing of Protector vascular embolic protection device in

porcine model at Mayo Clinic (device patent pending).

1999 Evaluation of ability of intramuscular growth factor to stimulate angiogenesis in rabbit hindlimb model at 30 and 60 days post-procedure. Sponsored by Sulzer Medical.

1999 Use of *Vessea* device to close porcine peripheral artery tears (patent #6,159,197) Sponsored by Phoenix Heart Center.

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AWARDS & HONORS:

Columbia/HCA Cardiovascular Management Network - 1998 Cardiologist of the Year

PATENTS:

1. Method and Apparatus for Treating Body Tissues and Bodily Fluids; Patent granted December 12, 2000 Number: 6,159,197
2. Hot Tip Catheter; Patent granted February 20, 2001 Number: 6,190,379
3. Embolism Prevention Device; Patent granted April 2, 2002 Number: 6,364,900
4. Catheter apparatus and Method for Arterializing a Vein; Patent granted October 15, 2002 Number 6,464,665
5. Methods and apparatus for treating body tissues and bodily fluid vessels; Patent granted October 15, 2002 Number: 6,464,681
6. Catheter for Thermal Evaluation of Arteriosclerotic Plaque; Patent granted March 25, 2003 Number: 6,536,949
7. Small Diameter Snare; Patent granted April 29, 2003 Number: 6,554,842

**EXHIBIT
B**

DISCLOSURES

**APPLICATION
SERIAL NO. 09/836,750**

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

EXHIBIT C

DEFINITIONS

EXHIBIT C

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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Growth factor: a substance (as a vitamin B₁₂ or an interleukin)
that promotes growth and especially cellular growth

EXHIBIT D

CLAIMS

EXHIBIT D

CLAIMS

Claim X: A method for growing a new portion of a pre-existing heart comprising the steps of: placing a growth factor in a body of a human patient and growing new muscle in said heart.

EXHIBIT E

PUBLICATIONS

EXHIBIT E

PUBLICATION INFORMATION SUMMARY

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Left Ventricular Electromechanical Mapping to Assess Efficacy of phVEGF165 Gene Transfer for Therapeutic Angiogenesis in Chronic Myocardial Ischemia	Vale	Circulation. 2000; 102:965-974	08/29/00	U.S.	Small incision (minithoracotomy) with syringe injection	VEGF (Gene form)	Repair of damaged portion of heart – Also pertains to new muscle growth
Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans	Strauer	Circulation. 2002; 106:1913-1918	10/08/02	Germany	Balloon catheter with injection	Bone Marrow Cells	Repair of dead portion of heart – also pertains to new muscle growth

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Viability and differentiation of autologous skeletal myoblast grafts in ischemic cardiomyopathy	Hagege	Lancet 2003 Feb 8; 361 (9356):491-492	2003	France	Injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle)
Autologous Cell Transplant Helpful in Ischemic Heart or Legs	Barclay	Medscape Medical News 2000 – Abstract from American Heart Association's 75 th Scientific Sessions on 11/18/02, Chicago	11/18/02	U.S.	Surgery with syringe injection	Bone Marrow Cells	Repair of damaged portion of heart – also pertains to new muscle growth
Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation	Pagani	J Am Coll Cardiol 2003 Mar 5; 41(5): 879-888	2003	U.S.	Surgery with syringe injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle and blood vessels)

Clinical Investigation and Reports

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow–derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12 \pm 7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.^{1,2} Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.³

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.⁴⁻⁷ Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow–derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.⁸⁻¹⁰ Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.¹¹⁻¹⁴ However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.¹⁵ Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.¹⁶ However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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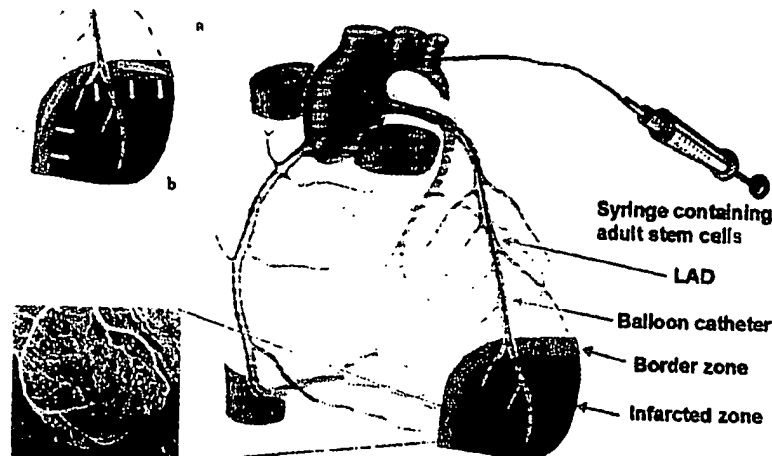


Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. *a*, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. *b*, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. *c*, A supply of blood flow exists within the infarcted zone.³⁵ The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.¹⁷ To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery ($n=4$), left circumflex coronary artery ($n=3$), or right coronary artery ($n=13$). Mean duration of infarct pain was 12 ± 10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty ($n=20$) and subsequent stent implantation ($n=19$). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β -blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (± 2) days after acute coronary angiography, bone marrow (~ 40 mL) was aspirated under local anesthesia from ilium of cell therapy patients ($n=10$). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O . For overnight

cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was $93 \pm 3\%$. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7 ; this consisted of $0.65 \pm 0.4\%$ AC133-positive cells and $2.1 \pm 0.28\%$ CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality ex vivo control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.¹⁸

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.¹⁹ All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2
Mononuclear bone marrow cells, n (×10 ⁷)	2.8±2.2

Values are mean±SD or number of patients. NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ²⁰¹thallium scintigraphy. The contractility index $P_{\text{max}}/\text{ESV}$ was calculated by dividing LV systolic pressure (P_{max}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, $P=0.005$). It was also significantly smaller compared with the standard therapy group after 3 months ($P=0.04$). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, $P=NS$). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², $P=0.016$, assessed by ²⁰¹thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

- (1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², $P=0.010$) and ejection fraction (from 51±14 to 53±13%, $P=NS$).
- (2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, $P=NS$) and end-systolic volume (from 82±26 to 67±21 mL, $P=0.011$). Radionuclide ventriculography was used to acquire the data.
- (3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, $P=NS$, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, $P=0.005$).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow-derived cells in experimental infarction.^{11–14,18,20–23} Moreover, trans-endothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.^{3,12,24–26} Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.^{15,16} Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.²⁷ Adult, mononuclear BMCs contain such stem and progenitor cells ($\leq 1\%$), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

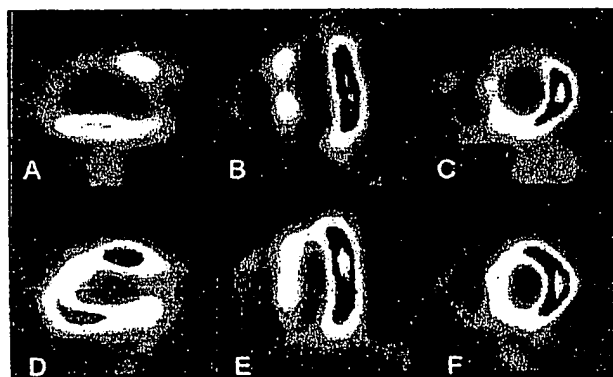


Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ^{201}Tl scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume index, mL/m ²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P ₂₀₀ /ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
^{201}Tl scintigraphy, cm ²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells¹³; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells²⁸; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.²⁹ Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of *in vitro* amplified hematopoietic stem or progenitor cells.³⁰

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ≈ 200 g) will flow per minute.^{31,32} This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).³¹ Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,³³ because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.³⁴ To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

- (1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.³⁵ Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

- (2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.³⁶ Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.
- (3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.³⁷⁻⁴⁰ Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility ($P_{1/2}/ESV$ and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

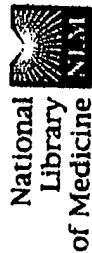
Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.⁴¹ None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12 ± 10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.⁴¹ In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angiogenesis and cardiomyogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy.

Hagege AA, Carrion C, Menasche P, Vilquin JT, Duboc D, Marolleau JP, Desnos M, Bruneval P.

Assistance Publique-Hopitaux de Paris, Department of Cardiology, Hopital Europeen Georges Pompidou and INSERM EMI-16, Necker-Paris V University, Paris, France. hagege@club-internet.fr

Autologous skeletal myoblast transplantation might improve postinfarction ventricular function, but graft viability and differentiation (ie, proof of concept) has not been shown. A 72-year-old man had autologous cultured myoblasts from his vastus lateralis injected to an area of transmural inferior myocardial infarction in non-reperfused scar tissue. He showed improvement in symptoms and left-ventricular ejection fraction. When he died 17.5 months after the procedure, the grafted post-infarction scar showed well developed skeletal myotubes with a preserved contractile apparatus. 65% of myotubes expressed the slow myosin isoform and 33% coexpressed the slow and fast isoforms (vs 44% and 0.6%, respectively, in skeletal muscle). Myoblast grafts can survive and show a switch to slow-twitch fibres, which might allow sustained improvement in cardiac function.

PMID: 12583951 [PubMed - indexed for MEDLINE]

myocardial tissue. These results establish the feasibility of myoblast transplants for myocardial repair in humans.

Publication Types:

- Clinical Trial
- Clinical Trial, Phase I

PMID: 12628737 [PubMed - indexed for MEDLINE]

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Autologous Cell Transplant Helpful in Ischemic Heart or Legs

Laurie Barclay, MD

Medscape Medical News 2002. © 2002 Medscape

Nov. 18, 2002 — Autologous cell transplantation may benefit ischemic hearts and legs, according to three presentations on Nov. 18 at the American Heart Association's 75th Scientific Sessions held in Chicago, Illinois. Two studies focused on injecting autologous bone marrow cells or autologous skeletal myoblasts into the scarred area of an infarcted heart. In another study, injecting autologous bone marrow into ischemic limbs led to new vessel growth, reducing the need for amputation.

"Bone marrow not only can differentiate into heart cells, but also smooth muscle cells, connective tissue cells and other types of cells to reconstitute the entire structure of a tissue," presenter Manuel Galinanes, MD, from the University of Leicester in the U.K., says in a news release. "The benefit [of transplanting bone marrow into scar tissue of the heart] could be seen only six weeks after injection."

In 14 patients with low ejection fraction post-myocardial infarction (MI), autologous bone marrow from the sternum was injected into scarred myocardium during nonemergency coronary artery bypass surgery. Heart wall motion measured with echocardiography improved within weeks of treatment, and improvements persisted for at least 10 months after treatment.

The regional wall motion score decreased significantly, reflecting less movement abnormality, from a mean score of 2.41 at baseline to 2.16 six weeks **after** treatment and 2.09 ten months **after** treatment. The global wall motion score also decreased significantly from 1.96 before surgery to 1.64 at six weeks, and stabilized at 1.65 **after** 10 months.

Although it is still unproven that bone marrow creates a new cellular infrastructure in **heart scar tissue**, "that is the only possible explanation," Galinanes says. "The ability to confirm the presence of scar tissue with dobutamine stress echo before surgery, and then confirm it again during surgery, told us that the affected area was dysfunctional and the abnormality was irreversible. We wanted to make sure that we were injecting the marrow into **dead tissue** to help ensure that the injection would not pose any serious risk to the patient."

If additional studies confirm safety and efficacy, Galinanes says that this treatment would be a welcome addition to the post-MI arsenal, which also includes gene therapy, growth factor therapy, and laser treatments.

In a multicenter trial supervised by the U.S. Food and Drug Administration, investigators safely transplanted 16 patients with autologous skeletal myoblasts injected into hearts severely damaged by MI or heart failure. Baseline left-ventricular ejection fraction was less than 30%. Eleven patients were undergoing coronary artery bypass surgery and five were having implantation of a left ventricular assist device. Myoblasts extracted from thigh muscle were grown in large quantities *in vitro* using a controlled cell expansion manufacturing process, and were injected in doses ranging from 10 million to 300 million cells.

"We have been able to **regenerate dead heart muscle**, or scar tissue, in the area of heart attack without increasing risk of death. Our findings will allow us to move forward with testing if the procedure can improve the contractility of the heart," says lead author Nabil Dib, MD, from the Arizona Heart Institute in Phoenix. "We found that the transplanted myoblasts survived and thrived in patients. Areas damaged by heart attack and cardiovascular disease showed evidence of repair and viability."

Twelve weeks **after** transplant, mean ejection fraction rates improved from 22.7% to 35.8%, or a 58% increase. Echocardiogram, magnetic resonance imaging, and positron emission tomography showed evidence of regeneration in the area of the graft. There were no significant adverse events related to the cell transplant procedure at nine-month follow-up.

The third study showed that bone marrow cells implanted into ischemic legs in patients with peripheral arterial disease (PAD) formed new blood vessels, increased blood flow, and prevented amputation.

"This is the first multicenter and double-blind clinical study to prove the clinical efficacy of growing new blood vessels (angiogenesis) using bone marrow cell transplantation," says lead author Hiroya Masaki, MD, PhD, from Kansai Medical University in Osaka, Japan.

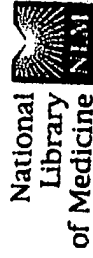
In this randomized trial, 45 patients with PAD received injections of autologous bone marrow mononuclear cells into the calf muscles. Compared with controls who received saline injections, patients who received bone marrow mononuclear cell transplants had a "striking" increase in new capillary formation and in newly visible collateral vessels.

Of 45 treated patients, 31 had an increase in ankle-brachial pressure index in the treated limbs, and 39 had decreased rest pain with improved treadmill endurance. Ischemic ulcers or gangrene healed in 21 of 28 treated limbs.

CD34-cells, which can develop into endothelial progenitor cells, expressed angiogenic growth factors including basic fibroblast growth factor, vascular endothelial growth factor, and angiopoietin-1. Although more research is needed to determine long-term efficacy and safety, "this new angiogenesis therapy using bone marrow cell transplantation may help many patients suffering with ischemic limbs," Masaki says.

AHA 75th Scientific Sessions: Abstracts 111623, 101758, 109801. Presented Nov. 18, 2002.

Reviewed by Gary D. Vagin, MD



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Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation.

Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, Dinsmore JH, Wright S, Aretz TH, Eisen HJ, Aaronson KD.

Section of Cardiac Surgery, University of Michigan, Ann Arbor, MI 48109, USA. fpagani@umich.edu

OBJECTIVES: We report histological analysis of hearts from patients with end-stage heart disease who were transplanted with autologous skeletal myoblasts concurrent with left ventricular assist device (LVAD) implantation. **BACKGROUND:** Autologous skeletal myoblast transplantation is under investigation as a means to repair infarcted myocardium. To date, there is only indirect evidence to suggest survival of skeletal muscle in humans. **METHODS:** Five patients (all male; median age 60 years) with ischemic cardiomyopathy, refractory heart failure, and listed for heart transplantation underwent muscle biopsy from the quadriceps muscle. The muscle specimen was shipped to a cell isolation facility where myoblasts were isolated and grown. Patients received a transplant of 300 million cells concomitant with LVAD implantation. Four patients underwent LVAD explant after 68, 91, 141, and 191 days of LVAD support (three transplant, one LVAD death), respectively. One patient remains alive on LVAD support awaiting heart transplantation. **RESULTS:** Skeletal muscle cell survival and differentiation into mature myofibers were directly demonstrated in scarred myocardium from three of the four explanted hearts using an antibody against skeletal muscle-specific myosin heavy chain. An increase in small vessel formation was observed in one of three patients at the site of surviving myotubes, but not in adjacent tissue devoid of engrafted cells. **CONCLUSIONS:** These findings represent demonstration of autologous myoblast cell survival in human heart. The implanted skeletal myoblasts formed viable grafts in heavily scarred human

EVIDENCE APPENDIX

ITEM NO. 21

**Supplemental Declaration of Dr. Heuser filed on February 17,
2004**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: E.C. Kemmerer, Ph.D.
)	
FILED: April 17, 2001)	
)	
FOR: METHOD FOR GROWING)	GROUP ART UNIT: 1646
MUSCLE IN A HUMAN HEART)	

SUPPLEMENTAL DECLARATION OF RICHARD HEUSER, M.D.

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. This Supplemental Declaration is submitted in addition to my previously submitted Declaration in this application, dated June 5, 2003, and makes no changes to such previous Declaration.
3. My Curriculum Vitae is attached as Exhibit A to my previous Declaration.
4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as read and understood by me in my previous Declaration. A copy of such disclosures is attached hereto as Supplemental Exhibit A.

5. I note that the disclosures referenced in above Paragraph 4 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new muscle in the heart.
6. I have read and understood the claims set forth in Supplemental Exhibit B and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.
7. Based upon above Paragraphs 4-6 and Paragraph 7 of my previous Declaration, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.
8. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Supplemental Exhibit B without need for resorting to undue experimentation. I have been informed that the Examiner has questioned the fact that dosages are not recited in the specification of the above-identified application in connection with the administration of cell growth factors to a human patient with use of intravenous or intraluminal techniques. Such techniques are the subject of claims 248-249 in above-mentioned Supplemental Exhibit B. In my opinion, dosages of cellular growth factors to achieve the above-mentioned heart muscle growth are a matter of routine medical practice, requiring only a reasonable degree of experimentation, depending upon such factors as extent of prior heart condition, size of patient, age of patient, health of patient, etc. Consequently, it is my opinion that the disclosure mentioned in Supplemental Exhibit A would enable a person skilled in the medical arts to practice the invention of claims 248-249 and predictably anticipate the results defined therein without need for resorting to undue experimentation.

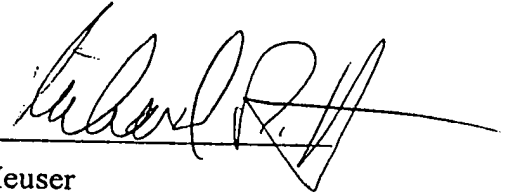
9. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date:

2/4/04


Richard Heuser

SUPPLEMENTAL EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

SUPPLEMENTAL EXHIBIT B

CLAIMS APPLICATION SERIAL NO. 09/836,750

- 236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new muscle and growing a new artery in said heart.
- 238. The method of claim 236, further comprising repairing a dead portion of said heart.
- 239. The method of claim 236, further comprising repairing a damaged portion of said heart.
- 240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.
- 241. The method of claim 240, wherein said genetic material comprises a gene.
- 242. The method of claim 241, wherein said gene comprises VEGF.
- 243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
- 244. The method of claim 243, wherein said growth factor comprises a cell
- 245. The method of claim 244, wherein said cell is multifactorial and non-specific.
- 246. The method of claim 245, wherein said cell comprises a stem cell.

- 247. The method of claim 236, wherein said growth factor is placed in said patient by injection.
- 248. The method of claim 247, wherein said injection is intravenous.
- 249. The method of claim 247, wherein said injection is intraluminal.
- 250. The method of claim 247, wherein said injection is intramuscular.
- 251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.
- 252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.
- 253. The method of claim 236, wherein said growth factor comprises a gene and a cell.

EVIDENCE APPENDIX

ITEM NO. 22

**2nd Supplemental Declaration of Dr. Heuser
filed on July 30, 2004**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: E.C. Kemmerer, Ph.D.
)	
FILED: April 17, 2001)	
)	GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING)	
MUSCLE IN A HUMAN HEART)	

**SECOND SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.**

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. This Second Supplemental Declaration is submitted in addition to my previous Declaration, dated June 5, 2003 and my Supplemental Declaration dated February 4, 2004. No changes are made to either of such previous Declarations.
3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my Declaration of June 5, 2003.
4. It is my understanding that the Examiner in charge of the above-identified patent

application, in an Office Action dated June 1, 2004 for related patent application Serial No. 09/794,456, questioned my qualification, for the first time, to render my previous opinions mentioned in above Paragraph 2. It is my further understanding that the basis for such questioning was that the Examiner noted that I did not report experience with cellular therapy. I desire to provide the information contained in following paragraph 5 so that the Examiner can consider such information in this application, as well.

5. I am currently Director of Cardiovascular Research at St. Joseph's Hospital and Medicine Center, and I serve as Clinical Professor of Medicine at University of Arizona College of Medicine. Over the past six years, I have worked in gene therapy, as well as muscle regeneration for the treatment of cardiomyopathy.

In my CV, you will note reference to work that was done with Sulzer Medical involving a rabbit hind limb model to stimulate peripheral vascular disease. I injected a growth mixture that included FGF, etc. into the hind limb model.

In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

6. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 17; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood in my previous Declaration and

Supplemental Declaration. A copy of such disclosures is attached hereto as Second Supplemental Declaration Exhibit A.

7. I note that the disclosures referenced in above Paragraph 6 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle in the heart.
8. I have read and understood the claims set forth in Second Supplemental Declaration Exhibit B and have been informed that such claims are currently presented in this application.
9. Based upon above Paragraphs 6-8, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be enabled to practice the method set forth in Second Supplemental Declaration Exhibit B and to predictably anticipate the results defined therein without need for resorting to undue experimentation.
10. I believe that one skilled in the medical arts, upon reading the disclosures in above , such as multifactorial and non-specific cells, Paragraph 6, would understand that cellular growth factors are included in such disclosures. Moreover, such skilled person would understand the disclosure on page 45 to be authored as an illustration of various modes of delivery of growth factors, whether they are genes or other genetic material; and that such skilled person would further understand that the disclosures on pages 45 and 46 describe genetic material to include appropriate cells and genes.

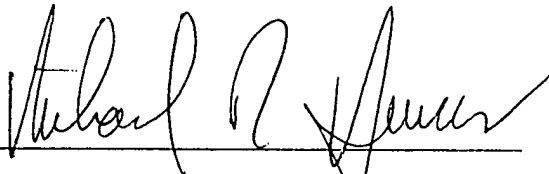
11. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: _____

7/18/04



Richard Heuser, M.D., F.A.C.C., F.A.C.P.

**SECOND
SUPPLEMENTAL
DECLARATION**

EXHIBIT A

DISCLOSURES

**SECOND SUPPLEMENTAL DECLARATION
EXHIBIT A**

**DISCLOSURES
APPLICATION SERIAL NO. 09/836,750**

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in

connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the

heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

**SECOND SUPPLEMENTAL
DECLARATION**

EXHIBIT B

CLAIMS

**SECOND SUPPLEMENTAL DECLARATION
EXHIBIT B**

**CLAIMS
APPLICATION SERIAL NO. 09/836,750**

236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.
238. The method of claim 236, further comprising repairing a dead portion of said heart.
239. The method of claim 236, further comprising repairing a damaged portion of said heart.
240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.
241. The method of claim 240, wherein said genetic material comprises a gene.
242. The method of claim 241, wherein said gene comprises VEGF.
243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
244. The method of claim 243, wherein said growth factor comprises a cell
245. The method of claim 244, wherein said cell is multifactorial and non-specific.
246. The method of claim 245, wherein said cell comprises a stem cell.

- 247. The method of claim 236, wherein said growth factor is placed in said patient by injection.
- 248. The method of claim 247, wherein said injection is intravenous.
- 249. The method of claim 247, wherein said injection is intraluminal.
- 250. The method of claim 247, wherein said injection is intramuscular.
- 251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.
- 252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.
- 253. The method of claim 236, wherein said growth factor comprises a gene and a cell.
- 254. A method of growing a new portion of a pre-existing organ comprising placing a growth factor in a body of a patient to grow new muscle in said organ.
- 255. The method of claim 254, wherein said organ comprises a heart.
- 256. The method of claim 255, wherein said new muscle comprises cardiac muscle and said growth factor comprises a stem cell.

EVIDENCE APPENDIX

ITEM NO. 23

**3rd Supplemental Declaration of Dr. Heuser
cited by Appellant as Exhibit I in the Appeal Brief
filed June 13, 2005**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: E.C. Kemmerer, Ph.D.
)	
FILED: April 17, 2001)	
)	GROUP ART UNIT: 1646
FOR: METHOD FOR GROWING)	
MUSCLE IN A HUMAN HEART)	

THIRD SUPPLEMENTAL DECLARATION
OF RICHARD HEUSER, M.D., F.A.C.C., F.A.C.P.

I, Richard Heuser, declare as follows:

1. I have offices at 500 West Thomas Road, Suite 900, Phoenix, Arizona 85013.
2. This Third Supplemental Declaration is submitted in addition to my previous Declaration, dated June 5, 2003; my Supplemental Declaration dated February 4, 2004; and my Second Supplemental Declaration dated July 18, 2004. No changes are made to any of such previous Declarations.
3. My Curriculum Vitae is attached as Exhibit A to my Declaration of June 5, 2003.
4. It is my understanding that the Examiner in charge of the above-identified patent application is also in the Examiner in charge of co-pending patent application Serial No. 09/794,456. In an Advisory Action dated November 26, 2004, for aforesaid Serial No. 09/794456, the Examiner further questioned my qualification to render my opinions in the three previous Declarations mentioned in above Paragraph 2. It is my further

understanding that the Examiner reviewed my U.S. Patent No. 6,190,379 and did not find mention of delivery of any substance to the myocardium nor the word "cell." Also, the Examiner questioned my role in the cell delivery portion of Bioheart's laboratory and clinical trials using skeletal muscle cultured and modified. I provide the following information to respond to the Examiner's newly raised questions.

5. Regarding, U.S. Patent No. 6,190,379, the following is stated in my Second Supplemental Declaration:

In my U.S. Patent No. 6,190,379 entitled "Hot Tip Catheter," I developed a technique to deliver radiofrequency (PMR). In the full embodiment of the patent, I discuss delivery of protein and/or muscle cells in the myocardium using the inventive technique.

By the above statement, I meant that the device shown in the patent has been used for the delivery of protein and/or muscle cells to the myocardium. At a presentation at the Angiogenesis Meeting in 1999 in Washington, D.C., we described this use of growth factors in a pig model with the development of neo vascularization. Moreover, I have had discussions with Bioheart regarding the use of my U.S. Patent No. 6,190,379 for delivery of cells.

Regarding my work at Bioheart, the following is stated in my Second Supplemental Declaration:

I have been involved as a member of the scientific advisory board with the world leader in cardiomyocyte regeneration, Bioheart, Miami Lakes, Florida. This company has been involved with laboratory and clinical trials using skeletal muscle cultured and modified. The sample is then delivered into the myocardium via a surgical or catheter approach.

To provide further information regarding the Examiner's questioning my involvement with Bioheart, I am a Scientific Advisory Board Member and in such role advise Bioheart throughout its pre-clinical and clinical work involving the delivery of skeletal muscle

cells into the myocardium. I am also an investigator with Bioheart's Phase 3 clinical trials in the United States. Such trials have not yet commenced.

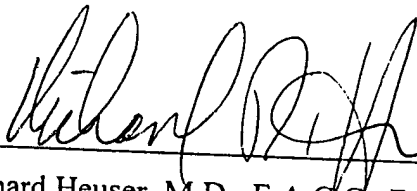
6. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date:

2/15/05


Richard Heuser, M.D., F.A.C.C., F.A.C.P.

EVIDENCE APPENDIX

ITEM NO. 24

Declaration of Dr. Andrew E. Lorincz filed on June 17, 2003

CERTIFICATE OF MAILING

I hereby certify that the attached DECLARATION OF ANDREW E. LORINCZ, M.D.
was delivered to the Assistant Commissioner for Patents by the undersigned from Arrow
Intellectual Property Services, 2001, Jefferson Davis Highway, Suite 602, Arlington, Virginia
22202, by hand carrying said DECLARATION to Art Unit 1646, Crystal Plaza 1, Tenth Floor,
Attention: Examiner Elizabeth C. Kemmerer this 17th day of June, 2003.

Dated: June 17, 2003

Ann Rutledge
Printed Name: Ann Rutledge

Docket No. ~~XXXXXX~~ 1000-10-CO1
Serial No. ~~XXXXXX~~ 09/836,750
Filed ~~XXXXXX~~ 04/17/01
Due Date: _____

ARROW INTELLECTUAL PROPERTY SERVICE

The Patent Office acknowledges, and has stamped hereon, the date of receipt
of the items check below:

- ☐ Transmittal Letter
- ☐ Application - Trademark
- ☐ Application - Patent Specification Total Pgs _____
- ☐ Total Claims _____ Ind. Claims _____ Total Pgs _____
- ☐ Fee: \$ _____
- ☐ Abstract Total Pgs _____
- ☐ Drawings: Formal _____ Informal _____ Total Pgs _____
- ☐ Declaration/Oath/Power of Attorney Total Pgs _____
- ☐ Assignment Fee: \$ _____
- ☐ Request for Non-Publication
- ☐ Information Disclosure Statement
- ☐ Form PTO-1449 References _____ Total No _____
- ☐ Request for Extension of Time Fee: \$ _____
- ☐ Amendment/Response
- ☒ ~~XXXXXX~~ Declaration of Andrew Lorincz
- ☐ Brief/Reply Brief/Notice of Appeal
- ☐ Fee-Base/Maintenance Fee: \$ _____
- ☐ Check No. _____ Fee: \$ _____
- ☐ _____
- ☐ _____

CCP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: E.C. Kemmerer, Ph.D.
)	
FILED: April 17, 2001)	
)	GROUP ART UNIT: 1646
FOR: METHOD AND APPARATUS)	
FOR INSTALLATION OF)	
DENTAL IMPLANT)	

DECLARATION OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 3628 Belle Meade Way, Mountain Brook, Alabama 35223.
2. My Curriculum Vitae is attached hereto as Exhibit A.
3. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. A copy of such disclosures is attached hereto as Exhibit B.
4. I note that the disclosures referenced in above Paragraph 3 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method of using a growth factor for growing muscle in a human heart.

5. I am aware of and have considered the definition of *growth factor* in the specification of the above-referenced patent application at page 20, line 10 through page 21, line 15. Such definition is set forth in Exhibit C along with a definition from the medical dictionary, MEDLINE plus: Merriam-Webster Medical Dictionary. A service of the U.S. NATIONAL LIBRARY OF MEDICINE and the NATIONAL INSTITUTES OF HEALTH. I find that the dictionary definition is consistent with that contained at page 20, line 10 through page 21, line 15 of the above-referenced patent application. I believe that both definitions are appropriate for use in the field of tissue growth and would be understood by one skilled in the medical arts. Accordingly, I am adopting and utilizing the definition contained in the patent application throughout this declaration.
6. I have read and understood the claims set forth in Exhibit D and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.
7. The materials included in attached Exhibit E illustrate that placement of a growth factor in a human patient causes muscle growth in a heart. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.
8. Based upon above Paragraphs 3-7, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.

9. Based upon above Paragraphs 3-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Exhibit D without need for resorting to undue experimentation.
10. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 6-9-03

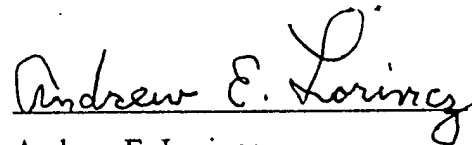

Andrew E. Lorincz

EXHIBIT A

**CURRICULUM
VITAE**

NAME: Andrew E. Lorincz, M.D.
TITLE: Professor of Pediatrics
University of Alabama at Birmingham
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3628 Belle Meade Way
Mountain Brook, Alabama 35223
Telephone: (205) 967-4678

BIRTH: 5/17/26 Chicago, Illinois

MARITAL STATUS: Married, 12/14/65 - Diane DeNyse Lorincz

EDUCATION:

1948-1952 University of Chicago, School of Medicine, M.D. Degree
1948-1950 University of Chicago, B.S. Degree (Anatomy & Biochemistry)
1946-1948 University of Chicago, Ph.B. Degree

POSTDOCTORAL EDUCATION:

Jan-Mar 1980 Lysosomal Storage Disease Laboratory, Eunice Kennedy Shriver Center,
Waltham, MA (Harvard), Visiting Scientist
1955-1956 LaRabida Jackson Park Sanitarium, University of Chicago,
Junior Staff Physician Department of Pediatrics, University of Chicago
Clinics Bob Roberts Memorial Hospital
1955-1958 Arthritis and Rheumatism Foundation Fellow
1954-1955 Benjamin J. Rosenthal Clinical and Research Fellow
1953-1954 Junior Assistant Resident
1952-1953 Intern

ACADEMIC APPOINTMENTS:

1996-present	Professor Emeritus, Department of Pediatrics
1984-1996	School of Public Health, University of Alabama at Birmingham, Professor
1971-1996	University of Alabama at Birmingham, Member of Graduate Faculty
1968-1996	University of Alabama at Birmingham, Professor of Pediatrics
1971-1984	Division of Engineering Biophysics, University of Alabama at Birmingham, Associate Professor
1968-1982	University of Alabama at Birmingham, Associate Professor of Biochemistry
1976- 1980	School of Optometry, University of Alabama at Birmingham, Professor Optometry
1971-1980	School of Nursing, University of Alabama at Birmingham, Clinical Associate Professor
1970-1980	Center for Developmental and Learning Disorders, University of Alabama at Birmingham, (A University Affiliated Facility for Developmental Disability), Director
1970-1976	School of Optometry, University of Alabama at Birmingham, Associate Professor of Pediatric Optometry
1966-1968	Medical Teaching and Research, Unit of the University of Florida at the Sunland Training Center, Gainesville, Florida, Director
1963-1968	Department of Surgery (Orthopaedics), University of Florida College of Medicine, Gainesville, Florida, Research Associate Professor
1962-1968	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Associate Professor
1959-1962	Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, Assistant Professor
1956-1959	Department of Pediatrics, University of Chicago School of Medicine, Chicago, Illinois, Instructor

PROFESSIONAL LICENSES - PHYSICIAN AND SURGEON:

5/26/69	State of Alabama
8/10/59	State of Florida (inactive)
9/22/54	State of Illinois (inactive)

SPECIALTY CERTIFICATION:

May 1958 American Board of Pediatrics, Diplomate

BOARDS, COMMITTEES AND CONSULTANTSIPS:

1994-present	Board member of The Mental Retardation and Developmental Disabilities, Health Care Authority of Jefferson County, Inc.
1991-present	Editorial Board for the <u>Annals of Clinical and Laboratory Science</u> , Member
1988-present	Medical Advisory Board of the National MPS Society, Member
1980-1986	<u>Mental Retardation</u> , Consulting Editor
1979-present	National Tay-Sachs and Allied Diseases Association, Scientific Advisory Committee, Member
1978-present	Mayor's Council of Disability Issues
1979-1984	Osteogenesis Imperfecta Foundation, Inc., Board Member Alabama O.I. Chapter
1974-1981	Child Mental Health Services, Inc., Birmingham, Alabama, Board Member
1977-1978	Elizabethtown Committee on Planning and Evaluation, Legislative Committee, State of Pennsylvania
1973-1975	Human Rights Committee for the Partlow State School and Hospital, Tuscaloosa, Alabama, Member - Federal Court Appointed
1971-1974	American Academy of Pediatrics, Committee on Children With Handicaps
1971-1973	<u>American Journal of Mental Deficiency</u> , Consulting Editor
1965-1973	Head Start, Medical Consultant
1967-1972	<u>Journal of Investigative Dermatology</u> , Editorial Consultant
1961-1968	Sunland Hospital, Orlando, Florida, Medical and Research Consultant
1965-1966	State of Florida Interagency Committee on Mental Retardation Planning, Co-Chairman, Mental Retardation Research Committee <u>Alabama Developmental Disabilities Planning Council</u>
1982-1984	Maternal and Child Health, Member of Advisory Committee
1979-1984	Member (Secretary, 1980; Vice Chairman, 1984)
1973-1979	Consultant

American Association of University Affiliated Facilities

1975-1978 American Association of University Affiliated Programs for the Developmentally Disabled, Board Member

American Association on Mental Retardation

1980,84,85	Prevention Committee, Chairman
1980-1982	Member of Council
1978-1980	Medicine Division and Member Executive Committee, Vice President

BOARDS, COMMITTEES AND CONSULTANTSHIPS: (CONTD)

Association of Retarded Citizens of Jefferson County

1990-present	Board Member
1975-1985	Board Member
1977-1978	Second Vice President
1980	Recipient of Distinguished Service Award

PROFESSIONAL SOCIETIES:

American Academy for Cerebral Palsy and Developmental Medicine, (Fellow)
American Academy on Mental Retardation (President Elect, 1975-76; President, 1976-77)
Emeritus Member
American Academy of Pediatrics (Fellow)
American Association for the Advancement of Science
American Association for Clinical Chemistry, Inc.
American Association on Mental Retardation (Fellow)-Life Member
American Chemical Society
American Federation for Clinical Research
American Medical Association
American Society for Human Genetics
American Society for Investigative Pathology
Association of Clinical Scientists
International Society for Mycoplasmaology
Jefferson County Pediatric Society
Society for Complex Carbohydrates
Society for Investigative Dermatology
Society for Pediatric Research
Society for Sigma Xi
Southern Society for Pediatric Research (President, 1964)

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1. "Modern Topics in Paediatric Dermatology", Verbov, J. (Editor), J.B. Lippincott, Philadelphia (Publisher), JAMA, 244:1269, 1980.
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EXHIBIT

B

DISCLOSURES

APPLICATION

SERIAL NO. 09/836,750

EXHIBIT B
DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

EXHIBIT C

DEFINITIONS

EXHIBIT C

DEFINITIONS

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

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Growth factor: a substance (as a vitamin B₁₂ or an interleukin)
that promotes growth and especially cellular growth

EXHIBIT D

CLAIMS

EXHIBIT D

CLAIMS

Claim X: A method for growing a new portion of a pre-existing heart comprising the steps of: placing a growth factor in a body of a human patient and growing new muscle in said heart.

EXHIBIT E
PUBLICATIONS

EXHIBIT E

PUBLICATION INFORMATION SUMMARY

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Left Ventricular Electromechanical Mapping to Assess Efficacy of phVEGF165 Gene Transfer for Therapeutic Angiogenesis in Chronic Myocardial Ischemia	Vale	Circulation. 2000; 102:965-974	08/29/00	U.S.	Small incision (minithoracotomy) with syringe injection	VEGF (Gene form)	Repair of damaged portion of heart – Also pertains to new muscle growth
Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans	Strauer	Circulation. 2002; 106:1913-1918	10/08/02	Germany	Balloon catheter with injection	Bone Marrow Cells	Repair of dead portion of heart – also pertains to new muscle growth

TITLE	AUTHOR	CITATION	DATE	AUTHOR COUNTRY	ROUTE OF ADMINISTRATION	GROWTH FACTOR ADMINISTERED	RESULT
Viability and differentiation of autologous skeletal myoblast grafts in ischemic cardiomyopathy	Hagege	Lancet 2003 Feb 8; 361 (9356):491-492	2003	France	Injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle)
Autologous Cell Transplant Helpful in Ischemic Heart or Legs	Barclay	Medscape Medical News 2000 – Abstract from American Heart Association's 75 th Scientific Sessions on 11/18/02, Chicago	11/18/02	U.S.	Surgery with syringe injection	Bone Marrow Cells	Repair of damaged portion of heart – also pertains to new muscle growth
Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation	Pagani	J Am Coll Cardiol 2003 Mar 5; 41(5): 879-888	2003	U.S.	Surgery with syringe injection	Skeletal Muscle Cells	Repair of dead portion of heart; Histological Proof (muscle and blood vessels)

Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans

Bodo E. Strauer, MD; Michael Brehm, MD; Tobias Zeus, MD; Matthias Köstering, MD; Anna Hernandez, PhD; Rüdiger V. Sorg, PhD; Gesine Kögler, PhD; Peter Wernet, MD

Background—Experimental data suggest that bone marrow-derived cells may contribute to the healing of myocardial infarction (MI). For this reason, we analyzed 10 patients who were treated by intracoronary transplantation of autologous, mononuclear bone marrow cells (BMCs) in addition to standard therapy after MI.

Methods and Results—After standard therapy for acute MI, 10 patients were transplanted with autologous mononuclear BMCs via a balloon catheter placed into the infarct-related artery during balloon dilatation (percutaneous transluminal coronary angioplasty). Another 10 patients with acute MI were treated by standard therapy alone. After 3 months of follow-up, the infarct region (determined by left ventriculography) had decreased significantly within the cell therapy group (from 30 ± 13 to $12 \pm 7\%$, $P=0.005$) and was also significantly smaller compared with the standard therapy group ($P=0.04$). Likewise, infarction wall movement velocity increased significantly only in the cell therapy group (from 2.0 ± 1.1 to 4.0 ± 2.6 cm/s, $P=0.028$). Further cardiac examinations (dobutamine stress echocardiography, radionuclide ventriculography, and catheterization of the right heart) were performed for the cell therapy group and showed significant improvement in stroke volume index, left ventricular end-systolic volume and contractility (ratio of systolic pressure and end-systolic volume), and myocardial perfusion of the infarct region.

Conclusions—These results demonstrate for the first time that selective intracoronary transplantation of autologous, mononuclear BMCs is safe and seems to be effective under clinical conditions. The marked therapeutic effect may be attributed to BMC-associated myocardial regeneration and neovascularization. (*Circulation*. 2002;106:1913-1918.)

Key Words: myocardial infarction ■ cell transplantation, intracoronary ■ angiogenesis ■ bone marrow ■ myogenesis

Remodeling of the left ventricle after myocardial infarction (MI) represents a major cause of infarct-related heart failure and death. This process depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue.^{1,2} Despite application of pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of cardiac muscle cells is able to replicate itself is encouraging but is still consistent with the concept that such regeneration is restricted to viable myocardium.³

In animal experiments, attempts to replace the necrotic zone by transplanting other cells (eg, fetal cardiomyocytes or skeletal myoblasts) have invariably succeeded in reconstituting heart muscle structures, ie, myocardium and coronary vessels. However, these cells fail to integrate structurally and do not display characteristic physiological functions.⁴⁻⁷ Another approach to reverse myocardial remodeling is to repair myocardial tissue by using bone marrow-derived cells. Bone

marrow contains multipotent adult stem cells that show a high capacity for differentiation.⁸⁻¹⁰ Experimental studies have shown that bone marrow cells (BMCs) are capable of regenerating infarcted myocardium and inducing myogenesis and angiogenesis; this leads in turn to amelioration of cardiac function in mice and pigs.¹¹⁻¹⁴ However, procedures based on this phenomenon remain largely uninvestigated in a human clinical setting.

An investigation of one patient receiving autologous skeletal myoblasts into a postinfarction scar during coronary artery bypass grafting revealed improvement of contraction and viability 5 months afterward.¹⁵ Autologous mononuclear BMCs transplanted in a similar surgical setting showed long-term improvement of myocardial perfusion in 3 of 5 patients and no change in 2 patients.¹⁶ However, such studies entail a surgical approach and are therefore associated with well-known perioperative risks. Moreover, this surgical procedure cannot be used with MI. We therefore looked for a nonsurgical, safer mode for transplanting autologous cells

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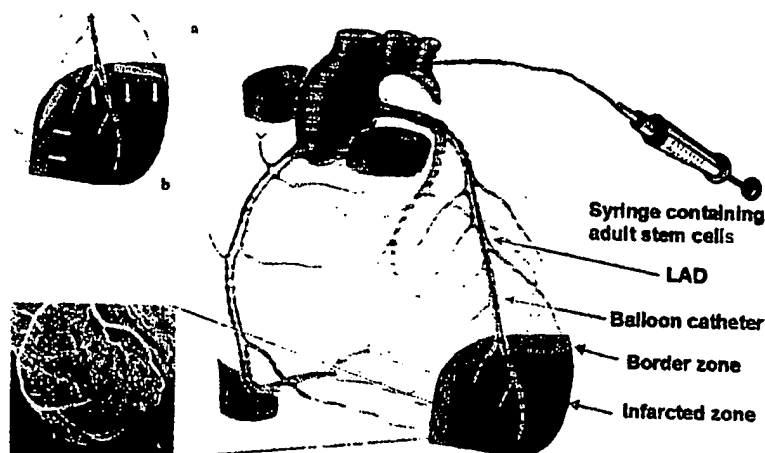


Figure 1. Procedure of cell transplantation into infarcted myocardium in humans. *a*, The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. It is then inflated and the cell suspension is infused at high pressure under stop-flow conditions. *b*, In this way, cells are transplanted into the infarcted zone via the infarct-related vasculature (red dots). Cells infiltrate the infarcted zone. Blue and white arrows suggest the possible route of migration. *c*, A supply of blood flow exists within the infarcted zone.³⁵ The cells are therefore able to reach both the border and the infarcted zone.

into postinfarction tissue. A pilot study from our group demonstrated that intracoronary transplantation of autologous mononuclear BMCs 6 days after MI was associated with a marked decrease in infarct area and an increase in left ventricular (LV) function after 3 and 6 months of follow-up.¹⁷ To confirm these results and validate this promising new therapy for MI, we established a clinical trial involving 20 patients for comparing the safety and bioefficacy of autologous BMC transplantation. All 20 patients underwent standard therapy, and 10 patients received additional intracoronary cell transplantation. All 20 patients were followed up for 3 months.

Methods

Patient Population

All 20 patients had suffered transmural infarction according to World Health Organization criteria with the involvement of the left anterior descending coronary artery ($n=4$), left circumflex coronary artery ($n=3$), or right coronary artery ($n=13$). Mean duration of infarct pain was 12 ± 10 hours before invasive diagnostics and therapy. Patients had to be <70 years old and were excluded if one of the following criteria were met: screening >72 hours after infarction, cardiac shock, severe comorbidity, alcohol or drug dependency, or excessive travel distance to the study center.

After right and left heart catheterization, coronary angiography, and left ventriculography, mechanical treatment was initiated with recanalization of the infarct-related artery by balloon angioplasty ($n=20$) and subsequent stent implantation ($n=19$). All patients were monitored in our intensive care unit, and no arrhythmogenic events or hemodynamic impairments were recorded in either patient group.

All 20 patients were briefed in detail about the procedure of BMC transplantation. Informed consent was obtained from 10 patients, who formed the cell therapy group, whereas 10 patients who refused additional cell therapy served as controls. The local ethics committee of the Heinrich-Heine-University, Düsseldorf, approved the study protocol. All procedures conformed to institutional guidelines.

Before taking part in rehabilitation programs, all patients left the hospital with standard medication consisting of acetylsalicylic acid, an ACE inhibitor, a β -blocker, and a statin.

Bone Marrow Aspiration, Isolation, and Cultivation

Seven (± 2) days after acute coronary angiography, bone marrow (~ 40 mL) was aspirated under local anesthesia from ilium of cell therapy patients ($n=10$). Mononuclear BMCs were isolated by Ficoll density separation on Lymphocyte Separation Medium (BioWhittaker) before the erythrocytes were lysed with H_2O . For overnight

cultivation, 1×10^6 BMCs/mL were placed in Teflon bags (Vuelife, Cell Genix) and cultivated in X-Vivo 15 Medium (BioWhittaker) supplemented with 2% heat-inactivated autologous plasma. The next day, BMCs were harvested and washed 3 times with heparinized saline before final resuspension in heparinized saline. Viability was $93 \pm 3\%$. Heparinization and filtration (cell strainer, FALCON) was carried out to prevent cell clotting and microembolization during intracoronary transplantation. The mean number of mononuclear cells harvested after overnight culture was 2.8×10^7 ; this consisted of $0.65 \pm 0.4\%$ AC133-positive cells and $2.1 \pm 0.28\%$ CD34-positive cells. All microbiological tests of the clinically used cell preparations proved negative. As a viability and quality *ex vivo* control, 1×10^5 cells grown in H5100 medium (Stem Cell Technology) were found to be able to generate mesenchymal cells in culture.

Intracoronary Transplantation of BMCs

Five to nine days after onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each. During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. PTCA thoroughly prevented the backflow of cells and at the same time produced a stop-flow beyond the site of the balloon inflation to facilitate high-pressure infusion of cells into the infarcted zone. Thus, prolonged contact time for cellular migration was allowed.¹⁸

Functional Assessment of Hemodynamics

After 3 months, all 20 patients were followed up by left heart catheterization, left ventriculography, and coronary angiography. Ejection fraction, infarct region, and regional wall movement of the infarcted zone during ejection were determined by left ventriculography. Ejection fraction was measured with Quantcor software (Siemens). To quantify infarction wall movement velocity, 5 axes were placed perpendicular to the long axis in the main akinetic or dyskinetic segment of the ventricular wall. Relative systolic and diastolic lengths were measured, and the mean difference was divided by the systolic duration (in seconds). To quantify the infarct region, the centerline method according to Sheehan was used.¹⁹ All hemodynamic investigations were obtained by two independent observers.

In the cell therapy group before and 3 months after cell transplantation, additional examinations for measuring hemodynamics and myocardial perfusion included dobutamine stress echocardiography, radionuclide ventriculography, catheterization of the right heart, and

TABLE 1. Baseline Characteristics of the Patients

Clinical Data	Cell Therapy	Standard Therapy	P
Characteristics			
No. of patients	10	10	...
Age, y	49±10	50±6	NS
Sex	Male	Male	...
Onset of infarction before angioplasty, h	10±8	13±11	NS
Coronary angiography			
No. of diseased vessels	1.7±0.9	2.1±0.7	NS
No. of patients with LAD/LCX/RCA as the affected vessel	4/1/5	0/2/8	...
No. of patients with stent implantation	9	10	...
Laboratory parameters			
Creatinine kinase, U/L	1138±1170	1308±1187	NS
Creatinine kinase-MB, U/L	106±72	124±92	NS
Bone marrow puncture after angioplasty, d	7±2
Mononuclear bone marrow cells, n (×10 ⁶)	2.8±2.2

Values are mean±SD or number of patients.
NS indicates not significant; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; and RCA, right coronary artery.

stress-redistribution-reinjection ²⁰¹thallium scintigraphy. The contractility index P_{100}/ESV was calculated by dividing LV systolic pressure (P_{100}) by end-systolic volume (ESV). Perfusion defect was calculated by scintigraphic bull's-eye technique. Each examination was performed according to standard protocols.

There were no complications or side effects determined in any patient throughout the diagnostic or therapeutic procedure or within the 3-month follow-up period.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when P was <0.05. Discrete variables were compared as rates, and comparisons were made by χ^2 analysis. Intra-individual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Comparison of nonparametric data between the two groups was performed with Wilcoxon test and Mann-Whitney test. Statistical analysis was performed with SPSS for Windows (version 10.1).

Results

Clinical data between the two groups did not differ significantly. The range of creatinine kinase levels was slightly but not significantly higher in the standard therapy group than it was in the cell therapy group (Table 1).

Comparison of the 2 groups 3 months after cell or standard therapy showed several significant differences in LV dynamics, according to the global and regional analysis of left ventriculogram. The infarct region as a percentage of hypokinetic, akinetic, or dyskinetic segments of the circumference of the left ventricle decreased significantly in the cell therapy group (from 30±13 to 12±7%, $P=0.005$). It was also significantly smaller compared with the standard therapy group after 3 months ($P=0.04$). Within the standard therapy group, only a statistically nonsignificant decrease from 25±8 to 20±11% could be seen. Wall movement velocity over the infarct region rose significantly in the cell therapy group (from 2.0±1.1 to 4.0±2.6 cm/s, $P=0.028$) but not in the standard therapy group (from 1.8±1.3 to 2.3±1.6 cm/s, $P=NS$). No significant difference was observed between the

two groups. Ejection fraction increased in both groups, albeit nonsignificantly (from 57±8 to 62±10% in the cell therapy group and from 60±7 to 64±7% in the standard therapy group) (Table 2).

Further significant improvement could also be seen on additional analysis of the cell therapy group alone. Perfusion defect was considerably decreased by 26% in the cell therapy group (from 174±99 to 128±71 cm², $P=0.016$, assessed by ²⁰¹thallium scintigraphy) (Figure 2). Parallel to the reduction in perfusion defect, improvement (Table 3) could also be seen in:

- (1) Cardiac function, as revealed by increase in stroke volume index (from 49±7 to 56±7 mL/m², $P=0.010$) and ejection fraction (from 51±14 to 53±13%, $P=NS$).
- (2) Cardiac geometry, as shown by decreases in both end-diastolic (from 158±20 to 143±30 mL, $P=NS$) and end-systolic volume (from 82±26 to 67±21 mL, $P=0.011$). Radionuclide ventriculography was used to acquire the data.
- (3) Contractility as evaluated by an increase in the velocity of circumferential fiber shortening (from 20.5±4.2 to 24.4±7.7 mm/s, $P=NS$, assessed by stress echocardiography) and by a marked increase in the ratio of systolic pressure to end-systolic volume (from 1.81±1.44 to 2.27±1.72 mm Hg/mL, $P=0.005$).

Discussion

The present report describes the first clinical trial of intracoronary, autologous, mononuclear BMC transplantation for improving heart function and myocardial perfusion in patients after acute MI. The results demonstrate that transplanted autologous BMCs may lead to repair of infarcted tissue when applied during the immediate postinfarction period. These results also show that the intracoronary approach of BMC transplantation seems to represent a novel

TABLE 2. Comparison of Cell Therapy and Standard Therapy Groups

	Cell Therapy	Standard Therapy	P
No. of patients	10	10	...
Infarct region as functional defect			
Hypokinetic, akinetic, or dyskinetic region at 0 mo, %	30±13	25±8	NS
Hypokinetic, akinetic, or dyskinetic region at 3 mo, %	12±7	20±11	0.04
P	0.005	NS	...
Contractility Indices			
Infarction wall movement velocity at 0 mo, cm/s	2.0±1.1	1.8±1.3	NS
Infarction wall movement velocity at 3 mo, cm/s	4.0±2.6	2.3±1.6	NS
P	0.028	NS	...
Hemodynamic data			
LV ejection fraction at 0 mo, %	57±8	60±7	NS
LV ejection fraction at 3 mo, %	62±10	64±7	NS
P	NS	NS	...

NS indicates not significant; 0 mo, zero months, which means the time of infarction; 3 mo, 3 months, which means the time of the follow-up examinations. All data were obtained according to analysis of left ventriculogram.

and effective therapeutic procedure for concentrating and/or depositing infused cells within the region of interest.

Neogenesis of both cardiomyocytes and coronary capillaries with some functional improvement has been shown recently by several investigators using bone marrow-derived cells in experimental infarction.^{11–14,18,20–23} Moreover, trans-endothelial migration from the coronary capillaries and incorporation of cells into heart muscle has been observed experimentally.^{3,12,24–26} Until now, clinical data only existed for the cell therapy of surgically treated chronic ischemic heart disease.^{15,16} Our aim was to transform the encouraging results from animal models to a safe clinical setting. The most crucial questions we had to address while designing and

realizing this trial were: (1) What cell population should we deliver? (2) Which application method is the most efficient? (3) When should the cells be transplanted?

In recent years, several laboratories have shown that environmentally dictated changes of fate (transdetermination) are not restricted to stem cells but may also involve progenitor cells at different steps of a given differentiation pathway (transdifferentiation). Moreover, mesenchymal stem cells may represent an ideal cell source for treating different diseases.²⁷ Adult, mononuclear BMCs contain such stem and progenitor cells ($\leq 1\%$), eg, mesodermal progenitor cells, hematopoietic progenitor cells, and endothelial progenitor cells. In several animal infarction models it has been shown that: (1) Bone marrow hemangioblasts contribute to the formation of new vessels; (2) bone marrow hematopoietic stem cells differentiate into cardiomyocytes, endothelium,

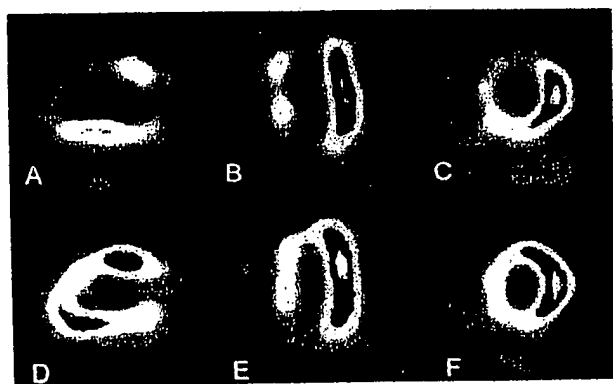


Figure 2. Improved myocardial perfusion of infarcted anterior wall 3 months after intracoronary cell transplantation subsequent to an acute anterior wall infarction detected by ²⁰¹thallium scintigraphy. The images on the left (A, D, sagittal) and in the middle (B, E) show the long axis, whereas those on the right (C, F, frontal) show the short axis of the heart. Initially the anterior wall, with green-colored apical and anterior regions, had reduced myocardial perfusion (A, B, C). Three months after cell transplantation the same anterior wall, now yellow in color, revealed a significant improvement in myocardial perfusion (D, E, F). All illustrations depict the exercise phase.

TABLE 3. Cardiac Function Analysis at 3-Month Follow-Up

	Before Cell Therapy	3 Months After Cell Therapy	P
No. of patients	10	10	...
Hemodynamic data			
LV ejection fraction, %	51±14	53±13	NS
Stroke volume Index, mL/m ²	49±7	56±7	0.010
Cardiac geometry			
LV end-diastolic volume, mL	158±20	143±30	NS
LV end-systolic volume, mL	82±26	67±21	0.011
Contractility Indices			
Circumferential fiber shortening, mm/s	20.5±4.2	24.4±7.7	NS
P _{ESV} /ESV, mm Hg/mL	1.81±1.44	2.27±1.72	0.005
Infarct region as perfusion defect			
²⁰¹ Thallium scintigraphy, cm ²	174±99	128±71	0.016

NS indicates not significant.

and smooth muscle cells⁸⁻¹³; (3) BMCs give rise to mesodermal progenitor cells that differentiate to endothelial cells²⁸; and (4) endothelial progenitors can transdifferentiate into beating cardiomyocytes.²⁹ Thus, several different fractions of mononuclear BMCs may contribute to the regeneration of necrotic myocardium and vessels. In order to utilize this large and perhaps heterogeneous regenerative potential, we decided to use all mononuclear cells from the bone marrow aspirate as a whole, rather than a subpopulation. No further expansion was performed because experimental data have revealed a dramatic decline in the homing capacity of in vitro amplified hematopoietic stem or progenitor cells.³⁰

The second question was how to deliver the cells most efficiently. When given intravenously, only a very small fraction of infused cells can reach the infarct region after the following injection: assuming a normal coronary blood flow of 80 mL/min per 100 g of LV weight, a quantity of 160 mL per left ventricle (assuming a regular LV mass of ~200 g) will flow per minute.^{31,32} This corresponds to only about 3% of cardiac output (assuming a cardiac output of 5000 mL/min).³¹ Therefore, intravenous application would require many circulation passages to enable infused cells to come into contact with the infarct-related artery. Throughout this long circulation and recirculation time, homing of cells to other organs could considerably reduce the numbers of cells dedicated to cell repair in the infarcted zone. Thus, supplying the entire complement of cells by intracoronary administration obviously seems to be advantageous for the tissue repair of infarcted heart muscle and may also be superior to intraventricular injection,³³ because all cells are able to flow through the infarcted and peri-infarcted tissue during the immediate first passage. Accordingly, by this intracoronary procedure the infarct tissue and the peri-infarct zone can be enriched with the maximum available amount of cells at all times.

As stem cells differentiate into more mature types of progenitor cells, it is thought that a special microenvironment in so-called niches regulates cell activity by providing specific combinations of cytokines and by establishing direct cellular contact. For successful long-term engraftment, at least some stem cells have to reach their niches, a process referred to as homing. Mouse experiments have shown that significant numbers of BMCs appear in liver, spleen, and bone marrow after intravenous injection.³⁴ To offer the BMCs the best chance of finding their niche within the myocardium, a selective intracoronary delivery route was chosen. Presumably, therefore, fewer cells were lost by extraction toward organs of secondary interest by this first pass-like effect. To facilitate transendothelial passage and migration into the infarcted zone, cells were infused by high-pressure injection directly into the necrotic area, and the balloon was kept inflated for 2 to 3 minutes; the cells were not washed away immediately under these conditions.

The time point for delivery was chosen as 7 to 8 days after infarction onset for the following reasons:

- (1) In dogs, infarcted territory becomes rich in capillaries and contains enlarged, pericyte-poor "mother vessels" and endothelial bridges 7 days after myocardial ischemia and reperfusion. Twenty-eight days later, a significant muscular vessel wall has already formed.³⁵ Thus, with such timing, cells may be able to reach the worst

damaged parts and at the same time salvage tissue. Transendothelial cell migration may also be enhanced because an adequate muscular coat is not yet formed.

- (2) Until now, only one animal study has attempted to determine the optimum time for cardiomyocyte transplantation to maximize myocardial function after LV injury. Adult rat hearts were cryoinjured and fetal rat cardiomyocytes were transplanted immediately, 2 weeks later, and 4 weeks later. The authors discussed the inflammatory process, which is strongest in the first days after infarction, as being responsible for the negative results after immediate cell transplantation, and they assumed that the best results seen after 2 weeks may have been due to transplantation before scar expansion.³⁶ Until now, however, no systematic experiments have been performed with BMCs to correlate the results of transplantation with the length of such a time delay.
- (3) Another important variable is the inflammatory response in MI, which seems to be a superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. In the first 48 hours, debridement and formation of a fibrin-based provisional matrix predominates before a healing phase ensues.³⁷⁻⁴⁰ Moreover, vascular endothelial growth factor is at its peak concentration 7 days after MI, and the decline of adhesion molecules (intercellular adhesion molecules, vascular cell adhesion molecules) does not take place before days 3 to 4 after MI. We assumed that transplantation of mononuclear BMCs within the "hot" phase of post-MI inflammation might lead them to take part in the inflammation cascade rather than the formation of functional myocardium and vessels.

Taking all of this into account, we can conclude that cell transplantation within the first 5 days after acute infarction is not possible for logistical reasons and is not advisable because of the inflammatory process. On the other hand, transplantation 2 weeks after infarction scar formation seems to reduce the benefit of cell transplantation. Although the ideal time point for transplantation remains to be defined, it is most likely between days 7 and 14 after the onset of MI, as in the present study.

This trial was designed as a phase I safety and feasibility trial, meaning that no control group is necessarily required. However, to validate the results, we correlated them with those obtained from 10 patients who refused to get additional cell therapy and thus received standard therapy alone. We are aware of the fact that such a comparison does not reach the power of a randomly allocated, blinded control group. However, the significant improvement with regard to infarct region, hemodynamics (stroke volume index), cardiac geometry (LV end-systolic volume), and contractility ($P_{1/2}/ESV$ and infarction wall movement velocity) did confirm a positive effect of the additional cell therapy because the changes observed in the standard therapy group failed to reach significance.

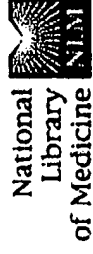
Another important factor for interpreting the results is time interval between onset of symptoms and revascularization of the infarct-related artery by angioplasty; this represents a crucial determinant of LV recovery. For patients with acute MI, it has

been shown that if the time interval is >4 hours, no significant changes in ejection fraction, regional wall motion, or ESV are observed after 6-month follow-up by echocardiography and angiography.⁴¹ None of our 20 patients was treated by angioplasty within 4 hours after onset of symptoms. Our average time interval was 12 ± 10 hours. Thus, PTCA-induced improvement of LV function can be nearly excluded; indeed, the only mild and nonsignificant changes within the standard therapy group are consistent with the above-mentioned data.⁴¹ In contrast, the cell therapy group showed considerable and significant improvement in the same parameters, which may be attributed to BMC-mediated coronary angiogenesis and cardiomyoneogenesis.

These results show that transplantation of autologous BMCs, as well as the intracoronary approach, represent a novel and effective therapeutic procedure for the repair of infarcted myocardium. For this method of therapy, no ethical problems exist, and no side effects were observed at any point of time. The therapeutic benefit for the patient's heart seems to prevail. However, further experimental studies, controlled prospective clinical trials, and variations of cell preparations are required to define the role of this new approach for the therapy of acute MI in humans.

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Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy.

Hagege AA, Carrion C, Menasche P, Vilquin JT, Duboc D, Marolleau JP, Desnos M, Bruneval P.

Assistance Publique-Hopitaux de Paris, Department of Cardiology, Hopital European Georges Pompidou and INSERM EMI-16, Necker-Paris V University, Paris, France. hagege@club-internet.fr

Autologous skeletal myoblast transplantation might improve postinfarction ventricular function, but graft viability and differentiation (ie, proof of concept) has not been shown. A 72-year-old man had autologous cultured myoblasts from his vastus lateralis injected to an area of transmural inferior myocardial infarction in non-reperused scar tissue. He showed improvement in symptoms and left-ventricular ejection fraction. When he died 17.5 months after the procedure, the grafted post-infarction scar showed well developed skeletal myotubes with a preserved contractile apparatus. 65% of myotubes expressed the slow myosin isoform and 33% coexpressed the slow and fast isoforms (vs 44% and 0.6%, respectively, in skeletal muscle). Myoblast grafts can survive and show a switch to slow-twitch fibres, which might allow sustained improvement in cardiac function.

PMID: 12583951 [PubMed - indexed for MEDLINE]

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myocardial tissue. These results establish the feasibility of myoblast transplants for myocardial repair in humans.

Publication Types:

- Clinical Trial
- Clinical Trial, Phase I

PMID: 12628737 [PubMed - indexed for MEDLINE]

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Autologous Cell Transplant Helpful in Ischemic Heart or Legs

Laurie Barclay, MD

Medscape Medical News 2002. © 2002 Medscape

Nov. 18, 2002 — Autologous cell transplantation may benefit ischemic hearts and legs, according to three presentations on Nov. 18 at the American Heart Association's 75th Scientific Sessions held in Chicago, Illinois. Two studies focused on injecting autologous bone marrow cells or autologous skeletal myoblasts into the scarred area of an infarcted heart. In another study, injecting autologous bone marrow into ischemic limbs led to new vessel growth, reducing the need for amputation.

"Bone marrow not only can differentiate into heart cells, but also smooth muscle cells, connective tissue cells and other types of cells to reconstitute the entire structure of a tissue," presenter Manuel Galinanes, MD, from the University of Leicester in the U.K., says in a news release. "The benefit [of transplanting bone marrow into scar tissue of the heart] could be seen only six weeks after injection."

In 14 patients with low ejection fraction post-myocardial infarction (MI), autologous bone marrow from the sternum was injected into scarred myocardium during nonemergency coronary artery bypass surgery. Heart wall motion measured with echocardiography improved within weeks of treatment, and improvements persisted for at least 10 months after treatment.

The regional wall motion score decreased significantly, reflecting less movement abnormality, from a mean score of 2.41 at baseline to 2.16 six weeks after treatment and 2.09 ten months after treatment. The global wall motion score also decreased significantly from 1.96 before surgery to 1.64 at six weeks, and stabilized at 1.65 after 10 months.

Although it is still unproven that bone marrow creates a new cellular infrastructure in heart scar tissue, "that is the only possible explanation," Galinanes says. "The ability to confirm the presence of scar tissue with dobutamine stress echo before surgery, and then confirm it again during surgery, told us that the affected area was dysfunctional and the abnormality was irreversible. We wanted to make sure that we were injecting the marrow into dead tissue to help ensure that the injection would not pose any serious risk to the patient."

If additional studies confirm safety and efficacy, Galinanes says that this treatment would be a welcome addition to the post-MI arsenal, which also includes gene therapy, growth factor therapy, and laser treatments.

In a multicenter trial supervised by the U.S. Food and Drug Administration, investigators safely transplanted 16 patients with autologous skeletal myoblasts injected into hearts severely damaged by MI or heart failure. Baseline left-ventricular ejection fraction was less than 30%. Eleven patients were undergoing coronary artery bypass surgery and five were having implantation of a left ventricular assist device. Myoblasts extracted from thigh muscle were grown in large quantities in vitro using a controlled cell expansion manufacturing process, and were injected in doses ranging from 10 million to 300 million cells.

"We have been able to regenerate dead heart muscle, or scar tissue, in the area of heart attack without increasing risk of death. Our findings will allow us to move forward with testing if the procedure can improve the contractility of the heart," says lead author Nabil Dib, MD, from the Arizona Heart Institute in Phoenix. "We found that the transplanted myoblasts survived and thrived in patients. Areas damaged by heart attack and cardiovascular disease showed evidence of repair and viability."

Twelve weeks after transplant, mean ejection fraction rates improved from 22.7% to 35.8%, or a 58% increase. Echocardiogram, magnetic resonance imaging, and positron emission tomography showed evidence of regeneration in the area of the graft. There were no significant adverse events related to the cell transplant procedure at nine-month follow-up.

The third study showed that bone marrow cells implanted into ischemic legs in patients with peripheral arterial disease (PAD) formed new blood vessels, increased blood flow, and prevented amputation.

"This is the first multicenter and double-blind clinical study to prove the clinical efficacy of growing new blood vessels (angiogenesis) using bone marrow cell transplantation," says lead author Hiroya Masaki, MD, PhD, from Kansai Medical University in Osaka, Japan.

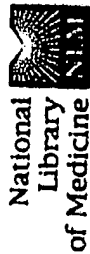
In this randomized trial, 45 patients with PAD received injections of autologous bone marrow mononuclear cells into the calf muscles. Compared with controls who received saline injections, patients who received bone marrow mononuclear cell transplants had a "striking" increase in new capillary formation and in newly visible collateral vessels.

Of 45 treated patients, 31 had an increase in ankle-brachial pressure index in the treated limbs, and 39 had decreased rest pain with improved treadmill endurance. Ischemic ulcers or gangrene healed in 21 of 28 treated limbs.

CD34-cells, which can develop into endothelial progenitor cells, expressed angiogenic growth factors including basic fibroblast growth factor, vascular endothelial growth factor, and angiopoietin-1. Although more research is needed to determine long-term efficacy and safety, "this new angiogenesis therapy using bone marrow cell transplantation may help many patients suffering with ischemic limbs," Masaki says.

AHA 75th Scientific Sessions: Abstracts 111623, 101758, 109801. Presented Nov. 18, 2002.

Reviewed by Gary D. Vogin, MD



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1: J Am Coll Cardiol 2003 Mar 5;41(5):879-88

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Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation.

Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, Dinsmore JH, Wright S, Aretz TH, Eisen HJ, Aaronson KD.

Section of Cardiac Surgery, University of Michigan, Ann Arbor, MI 48109, USA. fpagani@umich.edu

OBJECTIVES: We report histological analysis of hearts from patients with end-stage heart disease who were transplanted with autologous skeletal myoblasts concurrent with left ventricular assist device (LVAD) implantation. **BACKGROUND:** Autologous skeletal myoblast transplantation is under investigation as a means to repair infarcted myocardium. To date, there is only indirect evidence to suggest survival of skeletal muscle in humans. **METHODS:** Five patients (all male; median age 60 years) with ischemic cardiomyopathy, refractory heart failure, and listed for heart transplantation underwent muscle biopsy from the quadriceps muscle. The muscle specimen was shipped to a cell isolation facility where myoblasts were isolated and grown. Patients received a transplant of 300 million cells concomitant with LVAD implantation. Four patients underwent LVAD explant after 68, 91, 141, and 191 days of LVAD support (three transplant, one LVAD death), respectively. One patient remains alive on LVAD support awaiting heart transplantation. **RESULTS:** Skeletal muscle cell survival and differentiation into mature myofibers were directly demonstrated in scarred myocardium from three of the four explanted hearts using an antibody against skeletal muscle-specific myosin heavy chain. An increase in small vessel formation was observed in one of three patients at the site of surviving myotubes, but not in adjacent tissue devoid of engrafted cells. **CONCLUSIONS:** These findings represent demonstration of autologous myoblast cell survival in human heart. The implanted skeletal myoblasts formed viable grafts in heavily scarred human

EVIDENCE APPENDIX

ITEM NO. 25

**Supplemental Declaration of Dr. Andrew E. Lorincz
filed on February 17, 2004**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: E.C. Kemmerer, Ph.D.
)	
FILED: April 17, 2001)	
)	
FOR: METHOD FOR GROWING)	GROUP ART UNIT: 1646
MUSCLE IN A HUMAN HEART)	

SUPPLEMENTAL DECLARATION OF ANDREW E. LORINCZ, M.D.

I, Andrew E. Lorincz, declare as follows:

1. I reside at 13820 NW County Rd 235, Apt 8, Alachua, FL 32616-2098.
2. This Supplemental Declaration is submitted in addition to my previously submitted Declaration in this application, dated June 9, 2003, and makes no changes to such previous Declaration.
3. My Curriculum Vitae is attached as Exhibit A to my previous Declaration.
4. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as read and understood by me in my previous Declaration. A copy of such disclosures is attached hereto as Supplemental Exhibit A.

5. I note that the disclosures referenced in above Paragraph 4 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new muscle in the heart.
6. I have read and understood the claims set forth in Supplemental Exhibit B and have been informed that such claims will be presented to the Patent and Trademark Office in the near future.
7. Based upon above Paragraphs 4-6 and Paragraph 7 of my previous Declaration, it is my opinion that introducing a growth factor into a human patient will predictably cause new muscle growth in the heart of the patient.
8. Based upon above Paragraphs 4-6, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be able to practice the method set forth in Supplemental Exhibit B without need for resorting to undue experimentation. I have been informed that the Examiner has questioned the fact that dosages are not recited in the specification of the above-identified application in connection with the administration of cell growth factors to a human patient with use of intravenous or intraluminal techniques. Such techniques are the subject of claims 248-249 in above-mentioned Supplemental Exhibit B. In my opinion, dosages of cellular growth factors to achieve the above-mentioned heart muscle growth are a matter of routine medical practice, requiring only a reasonable degree of experimentation, depending upon such factors as extent of prior heart condition, size of patient, age of patient, health of patient, etc. Consequently, it is my opinion that the disclosure mentioned in Supplemental Exhibit A would enable a person skilled in the medical arts to practice the invention of claims 248-249 and predictably anticipate the results defined therein without need for resorting to undue experimentation.

9. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2-3-04

Andrew E. Lorincz
Andrew E. Lorincz

SUPPLEMENTAL EXHIBIT A
DISCLOSURES
APPLICATION SERIAL NO. 09/836,750

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of “hard tissue” or bone and “soft tissues” like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound,

by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ or tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in connection with any of the implant techniques of the invention, it is understood that a cell

nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles

and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

SUPPLEMENTAL EXHIBIT B

CLAIMS **APPLICATION SERIAL NO. 09/836,750**

236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new muscle and growing a new artery in said heart.
238. The method of claim 236, further comprising repairing a dead portion of said heart.
239. The method of claim 236, further comprising repairing a damaged portion of said heart.
240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.
241. The method of claim 240, wherein said genetic material comprises a gene.
242. The method of claim 241, wherein said gene comprises VEGF.
243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
244. The method of claim 243, wherein said growth factor comprises a cell
245. The method of claim 244, wherein said cell is multifactorial and non-specific.
246. The method of claim 245, wherein said cell comprises a stem cell.

- 247. The method of claim 236, wherein said growth factor is placed in said patient by injection.
- 248. The method of claim 247, wherein said injection is intravenous.
- 249. The method of claim 247, wherein said injection is intraluminal.
- 250. The method of claim 247, wherein said injection is intramuscular.
- 251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.
- 252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.
- 253. The method of claim 236, wherein said growth factor comprises a gene and a cell.

EVIDENCE APPENDIX

ITEM NO. 26

**2nd Supplemental Declaration of Dr. Andrew E. Lorincz
filed on July 30, 2004**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia)	
)	
SERIAL NO.: 09/836,750)	EXAMINER: E.C. Kemmerer, Ph.D.
)	
FILED: April 17, 2001)	
)	
FOR: METHOD FOR GROWING)	GROUP ART UNIT: 1646
MUSCLE IN A HUMAN HEART)	

**SECOND SUPPLEMENTAL DECLARATION
OF ANDREW E. LORINCZ, M.D.**

I, Andrew E. Lorincz, declare as follows:

1. I reside at 13820 NW County Rd 235, Apt 8, Alachua, FL 32616-2098.
2. This Second Supplemental Declaration is submitted in addition to my previous Declaration dated June 5, 2003 and my Supplemental Declaration dated February 3, 2004. No changes are made to either of such previous Declarations.
3. My Curriculum Vitae (hereinafter "CV") is attached as Exhibit A to my previous Declaration.
4. It is my understanding that the Examiner in charge of the above-identified patent application, in an Office Action dated June 1, 2004 for related patent application Serial No. 09/794,456, questioned my qualification, for the first time, to render my previous opinions mentioned in above Paragraph 2. It is my further understanding that the basis for such questioning was that the Examiner noted that I did not report experience with cellular therapy. I desire to provide the

information contained in following paragraph 5 so that the Examiner can consider such information in this application, as well.

5. In addition to the qualifications set forth in my CV, I am familiar with stem cell technology, including bone marrow preparation.
6. I have read and understood the disclosures of the above-referenced patent application at page 20, line 10 through page 21, line 15; and page 44, line 19 through page 46, line 16. Such disclosures are the same as I read and understood in my previous Declaration and Supplemental Declaration. A copy of such disclosures is attached hereto as Second Supplement Declaration Exhibit A.
7. I note that the disclosures referenced in above Paragraph 6 relate to using a growth factor for promoting the growth of soft tissue and, more specifically, to a method which may use such growth factors for growing a new portion of a human heart by growing new cardiac muscle in the heart.
8. I have read and understood the claims set forth in Second Supplemental Declaration Exhibit B and have been informed that such claims are currently presented in this application.
9. Based upon above Paragraphs 6-8, it is my opinion that one skilled in the medical arts, armed with the knowledge in such paragraphs, would be enabled to practice the method set forth in Second Supplemental Declaration Exhibit B and to predictably anticipate the results defined therein without need for resorting to undue experimentation.
10. I believe that one skilled in the medical arts, upon reading the disclosures in above Paragraph 6, would understand that cellular growth factors, such as multifactorial and non-specific cells, are included in such disclosures. Moreover,

such skilled person would understand the disclosure on page 45 to be authored as an illustration of various modes of delivery of growth factors, whether they are genes or other genetic material; and that such skilled person would further understand that the disclosures on pages 45 and 46 describe genetic material to include appropriate cells and genes.

11. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 7-19-04

Andrew E. Lorincz, M.D.
Andrew E. Lorincz, M.D.

**SECOND
SUPPLEMENTAL
DECLARATION**

EXHIBIT A

DISCLOSURES

**SECOND SUPPLEMENTAL DECLARATION
EXHIBIT A**

**DISCLOSURES
APPLICATION SERIAL NO. 09/836,750**

PAGE 20, LINE 10 – PAGE 21, LINE 15

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue, in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic (FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors and the carrier, at a desired location in the body. Time release capsules, granules, or

other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected *in vivo* chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

PAGE 44, LINE 19 – PAGE 46, LINE 16

Genetic material comprising a portion of a gene, a gene, genes, a gene product (i.e., a composition a gene causes to be produced like, for example, an organ-producing growth factor), growth factor, or an ECM (extracellular matrix) can be used in or on the body to grow an organ to tissue. For example, the vascular epithelial growth factor gene (VEGF) or its growth factor equivalent can be inserted into the body to cause an artery to grow. When insertion of a gene, portion of a gene, gene product, growth factor, or ECM *in vivo* or *ex vivo* is referred to herein in

connection with any of the implant techniques of the invention, it is understood that a cell nutrient culture(s), physiological nutrient culture(s), carrier (s), enhancer(s), promoter(s), or any other desired auxiliary component(s) can be inserted with the gene or at the same location as the gene, growth factor, ECM, etc.

An artery is an organ from the circulatory system. An artery can be grown in the heart, legs, or other areas by injecting a gene or other genetic material into muscle at a desired site. Size, vascularity, simplicity of access, ease of exploitation, and any other desired factors can be utilized in selecting a desired site. The gene is one of several known VEGF genes which cause the production of vascular endothelial growth factors. Several VEGF genes which produce vascular endothelial growth factors are believed to exist because nature intends for there to be several pathways (i.e., genes) which enable the production of necessary growth factors. The existence of several pathways is believed important because if one of the genes is damaged or inoperative, other similar genes can still orchestrate the production of necessary growth factors. VEGF genes are used by the body to promote blood vessel growth. VEGF genes are assimilated (taken in) by muscle cells. The genes cause the muscle cells to make a VEGF protein which promotes the growth of new arteries. VEGF proteins can be made in a lab and injected into a patient intravenously, intraluminally, or intramuscularly to promote the growth of an artery. Or, the genes (or other genetic material) can be applied with an angioplasty balloon, with the assistance of a vector, or by any other method.

It is not always desirable to grow a completely new organ. Sometimes growing a portion of an organ is desirable. For example, in some heart attacks or strokes, a portion of the heart or brain remains viable and a portion dies. An injection of a gene to form cardiac muscle and/or an injection of a gene to form an artery can be utilized to revive or replace the dead portion of the

heart. The dead portion of the heart may (or may not) be used as a matrix while the new muscles and vessels grow. Thus, in this example, a partial new organ is grown in a pre-existing organ. A pacemaker may (or may not) be necessary. A second injection of a gene may (or may not) be necessary to stop cardiac muscle growth once it is completed. Portions of organs throughout the body can similarly be repaired or replaced. It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ.

The size and shape limitation of the desired structure can come from a containment and boundary contact inhibition phenomenon or by a chemical inhibition.

A variation on the theme of growing a portion of an organ is as follows: a portion of a heart dies. The pericardium is utilized as a scaffold and seeded with cells and/or genes to grow new muscle, and genes (or other genetic material) to grow new arteries. Immediately adjacent the dead cardiac muscle, onto or into the pericardium, the appropriate cells, genes, and/or growth factors (or other genetic material) are placed. Once the new muscle and blood vessels have grown, the function specific tissue can be applied to the damaged portion of the heart and paced, if necessary, to augment cardiac action. If the surgeon desires, the dead muscle can be removed and the new muscle and blood vessels can be surgically rotated into the excised region and secured. This probably can be done endoscopically. In essence, the pericardium is utilized to allow the new muscle wall to grow. The new muscle wall is then transplanted into the damaged heart wall. This procedure utilizes the body as a factor to grow an organ and/or tissue, after which the organ and/or tissue is transplanted to a desired region. On the other hand, the new muscle wall may integrate itself into the old wall and not require transplantation.

**SECOND SUPPLEMENTAL
DECLARATION**

EXHIBIT B

CLAIMS

**SECOND SUPPLEMENTAL DECLARATION
EXHIBIT B**

**CLAIMS
APPLICATION SERIAL NO. 09/836,750**

236. A method of growing a new portion of a pre-existing heart comprising the steps of placing a growth factor in a body of a human patient and growing new cardiac muscle and growing a new artery in said heart.
238. The method of claim 236, further comprising repairing a dead portion of said heart.
239. The method of claim 236, further comprising repairing a damaged portion of said heart.
240. The method of claim 236, wherein said growth factor comprises genetic material selected from the group consisting of a portion of a gene, a gene, a gene product, and an extracellular matrix.
241. The method of claim 240, wherein said genetic material comprises a gene.
242. The method of claim 241, wherein said gene comprises VEGF.
243. The method of claim 236, wherein said growth factor comprises a member selected from the group consisting of cells, cellular products, and derivatives of cellular products.
244. The method of claim 243, wherein said growth factor comprises a cell
245. The method of claim 244, wherein said cell is multifactorial and non-specific.
246. The method of claim 245, wherein said cell comprises a stem cell.

- 247. The method of claim 236, wherein said growth factor is placed in said patient by injection.
- 248. The method of claim 247, wherein said injection is intravenous.
- 249. The method of claim 247, wherein said injection is intraluminal.
- 250. The method of claim 247, wherein said injection is intramuscular.
- 251. The method of claim 236, wherein said growth factor is placed in said patient by a carrier.
- 252. The method of claim 251, wherein said carrier comprises an angioplasty balloon.
- 253. The method of claim 236, wherein said growth factor comprises a gene and a cell.
- 254. A method of growing a new portion of a pre-existing organ comprising placing a growth factor in a body of a patient to grow new muscle in said organ.
- 255. The method of claim 254, wherein said organ comprises a heart.
- 256. The method of claim 255, wherein said new muscle comprises cardiac muscle and said growth factor comprises a stem cell.

EVIDENCE APPENDIX

ITEM NO. 27

**Final Office Action dated September 22, 2006
(page 22, first paragraph)**



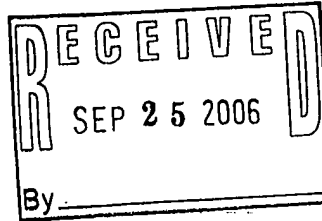
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/836,750	04/17/2001	James P. Elia	1000-10-C01	7239

7590 09/22/2006

Gerald K. White
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EXAMINER

KEMMERER, ELIZABETH

ART UNIT PAPER NUMBER

1646

DATE MAILED: 09/22/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary

Application No.

09/836,750

Applicant(s)

ELIA, JAMES P.

Examiner

Elizabeth C. Kemmerer, Ph.D.

Art Unit

1646

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 26 June 2006.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 6-236, 238-253 and 256-287 is/are pending in the application.
- 4a) Of the above claim(s) 6-235 and 240-242 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 236, 238, 239, 243-253 and 257-287 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
- Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
- Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
- 1 ☐ Certified copies of the priority documents have been received.
- 2 ☐ Certified copies of the priority documents have been received in Application No. _____.
- 3 ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☐ Information Disclosure Statement(s) (PTO/SB/08)
- Paper No(s)/Mail Date _____

- 4) ☐ Interview Summary (PTO-413)
- Paper No(s)/Mail Date _____
- 5) ☐ Notice of Informal Patent Application
- 6) ☐ Other: _____

DETAILED ACTION

Status of Application, Amendments, And/Or Claims

The amendment received 26 June 2006 has been entered in full. Claims 1-5, 237, and 254-256 are canceled. Claims 6-235 and 240-242 remain withdrawn from consideration as being directed to a non-elected invention. Claims 236, 238, 239, 243-253, and 257-287 are under examination.

The fourth supplemental declaration of Dr. Heuser under 37 CFR 1.132 and third supplemental declaration of Dr. Lorincz under 37 CFR 1.132 submitted with the response have been entered. A copy of the third supplemental declaration of Dr. Heuser under 37 CFR 1.132 has also been received.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

As an initial matter, it is noted that Applicant comments upon alleged procedural errors. The record has been reviewed and no errors in procedure have been noted. Therefore, these comments will no longer be addressed further.

35 U.S.C. § 112, First Paragraph, New Matter

Claims 248, 249, 252, and 274-279 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a new matter

rejection. The specification, as originally filed, does not contain support for intravenous, intraluminal, or angioplasty delivery of cells.

Applicant's arguments (pp. 41-51, amendment received 26 June 2006) have been fully considered but are not found to be persuasive for the following reasons.

Applicant points to p. 45, line 1 to p. 46, line 16 for supporting language. This is not found to be persuasive because intravenous, intraluminal, and angioplasty delivery are described as being useful for genes, proteins, or other genetic material, but not for cells. The specification does not include cells in its discussion of "genetic material." For example, p. 31, lines 11-13 state, "...the genetic material comprises comparable artificially produced genes, or genes harvested from other human beings or animals."

Applicant argues that the prior art teaches angioplasty delivery of cells, and thus the new matter rejection with regard to angioplasty balloon delivery of cells is incorrect. This has been fully considered but is not found to be persuasive because written description support for claimed subject matter must be in the specification as originally filed.

Applicant relies on pp. 20, 21, 46, 45, and 44 of the specification for their assertion that "genetic material" includes cells. This has been fully considered but is not found to be persuasive because these sections do not clearly indicate that "genetic material" includes cells. The specification defines "growth factors" as comprising cells, but does not define "genetic material" as comprising cells. For example, p. 31, lines 11-13. of the specification states "...the genetic material comprises comparable artificially produced genes, or genes harvested from other human beings or animals." Page 32,

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lines 8-9 state "genetic material can comprise comparable artificially produced genes or genes removed from another animal or otherwise generated." Page 35, line 4 clearly distinguished between growth factors (defined as encompassing cells) and genetic material: "genetic material plus growth factor(s) are implanted..." Page 35, lines 12-14 states "Genetic material is well conserved in nature. The *Drosophila* eyeless gene (*ey*), the mouse small *ey* gene (*pax-6*), and the Aniridia gene in humans are all homologous." Page 36, lines 25-26 state "Genes control structure and function. A gene or a bit of genetic material may act as a master control gene..." Clearly, the specification uses "genetic material" as pertaining to nucleic acids such as genes. It is also noted that one skilled in the art would only interpret "assistance of a vector," recited in the same sentence that uses "genetic material," as only applying to nucleic acids (genes or RNA or cDNA, etc.).

Applicant points to Capon v. Eshhar v. Dudas, 03-1480-1481 (CAFC 2005) as controlling precedent that 112 does not require recitation in the specification of features already known by workers in the technological field to which the invention is directed. Applicant urges that the examiner's distinction over Capon regarding products or method steps is inapt. This is not found to be persuasive because the instant fact pattern is still found to be distinct from that in the case law cited by Applicant. Capon v. Eshhar, 76 USPQ2d 1078 (CAFC 2005) concerns whether or not claims to chimeric DNA molecules are adequately described by a generic description. The issue here is not whether or not workers in this technology already knew the features of the cells recited in the claims; rather, the issue is that the instant specification did not set forth

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contemplation of a method step wherein cells were administered intravenously, intraluminally, or via angioplasty.

Applicant argues that the examiner's reliance on Lockwood v. American Airlines, Inc., 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997) is inappropriate since it also concerns products and not methods, and thus is contradictory to the examiner's position regarding Capon. This has been fully considered but is not found to be persuasive. The issue in Capon speaks to the relevance of what the skilled artisan already knew about cells. However, there was no question of whether or not the cells were set forth in the specification as part of the invention. Lockwood discusses how a specification can show possession of an invention. In the instant case, it does not appear that the originally filed specification set forth contemplation of the administration of cells intravenously, intraluminally, or via angioplasty balloon as being part of the invention. At best, the case law may be somewhat contradictory, and thus is an issue for the Board of Appeals to determine.

Applicant refers to the supplemental declarations of Drs. Heuser and Lorincz, the fourth supplemental declaration of Dr. Heuser, and the third supplemental declaration of Dr. Lorincz, specifically, point 10 of each. Applicant refers to the second supplemental declarations of Drs. Heuser and Lorincz, paragraphs 6 and 10. The declarations have been reviewed again. It is the opinion of Drs. Heuser and Lorincz that the specification's use of the term "genetic material" includes cells. Such constitutes evidence relevant to the issue. However, the specification, for example at pages 31, 32, 35, and 36, uses the term "genetic material" to describe genes. Furthermore, the art

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clearly uses the term "genetic material" to mean nucleic acids, not cells. For example, the textbook definition of genetic material set forth in Glossary of Genetics and Cytogenetics (fourth edition, 1976, Rieger et al., eds., p. 237) is provided in Appendix A.

The definition for genetic material is:

"the carrier of primary → genetic information: single or double-stranded → deoxyribonucleic acid (single in some, double in most bacteriophages, bacteria and higher organisms), or → ribonucleic acid (in RNA-viruses). G. M. must fulfill at least two fundamental functions: 1. serve as a template for its own → replication ("autocatalytic function"), 2. provide a template for the synthesis of other classes of macromolecules (specifically proteins), i.e., supply the structural and regulatory information it contains to the protein-synthesizing machinery of the cell ("heterocatalytic function").

Thus, it is a basic tenet of biology that cells do not constitute genetic material, they contain genetic material. It is noted that citing this reference does not constitute a new grounds of rejection. Rather, it is supporting a rejection of record.

Applicant refers to a definition from Wikipedia and pages from a publication called "The Cell Nucleus" to support their assertion that cells contain genetic information. The examiner agrees completely as this is the crux of the issue. Cells contain genetic material, they do not constitute genetic material. The Wikipedia definition, in fact, clearly supports this point.

Applicant argues that the examiner's indication that the specification defines "growth factors" as a genus comprising cells is fatal to the rejection, since p. 46, line 7 indicates that growth factors are a type of genetic material. This is not found to be persuasive. The issue of whether or not the term "growth factors" includes cells was a difficult issue, since the specification contained contradictory statements. For example,

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the line referred to by Applicant seems to indicate that cells are separate from growth factors, yet other portions of the specification list cells as belonging to the genus "growth factors." Therefore, the sentence relied upon by Applicant is inherently contradictory.

Applicant urges that the rejection is incorrect when taking the restriction requirement into consideration. Such has been considered but is not found to be persuasive because the elected invention is directed to administration of cells intravenously, intraluminally, or via angioplasty balloon. The specification does not support this concept by an adequate written description.

Applicant concludes that the rejection is improper and hypertechnical in view of the case law and the numerous 132 declarations by Drs. Heuser and Lorincz. This has been fully considered but is not found persuasive for the reasons cited above.

Specifically, statements at pp. 31, 32, 35, and 36 of the specification indicate that cells are not considered part of the term "genetic material." Also, the literature clearly indicates that cells *contain* genetic material, but do not *constitute* genetic material (see Appendix A, and the Wikipedia definition submitted by Applicant).

35 U.S.C. § 112, First Paragraph, Enablement

Claims 236, 238, 239, 243-253, and 257-287 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly

connected, to make and/or use the invention. The basis of this rejection is of record, but is re-printed here as per Applicant's request.

The claims require formation of a "new" artery. Applicant has defined a new artery as an organ comprising two or more kinds of tissues joined into one structure that has a certain task in the circulatory system. In Applicant's remarks section of the amendment received 17 February 2004, Applicant appears to imply that the "new artery" recited in the claims must be formed *de novo*, and not merely repair, growth or re-direction of an existing artery. See the discussion regarding fusion versus formation of new cells.

The courts have determined several factors to be considered in making a determination of whether or not undue experimentation would have been required of the skilled artisan to make and use the claimed invention (*In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988)). These are:

- 1) quantity of experimentation required,
- 2) amount of direction/guidance presented in the specification,
- 3) presence or absence of working examples,
- 4) nature of the invention,
- 5) state of the prior art,
- 6) level of skill of those in the art,
- 7) predictability, and
- 8) breadth of the claims.

1) In the instant case, the quantity of experimentation required would be very large. Applicant's attention is directed to pp. 1916 to 1918 of Strauer (of record, 2002, Circulation 106:1913-1918), who review the crucial questions that had to be addressed while designing and realizing their trial of administering stem cells to human patients to repair damaged heart tissue. These included decisions regarding what cell population to use, what delivery method to use, and when cells should be transplanted. As can be seen from pp. 1916-1918, these were not simple or routine matters and involved great quantities of experimentation. In fact, one can see that the determinations of these details involved the act of invention.

2) The specification provides no guidance along the lines of the details worked out by Strauer. The specification broadly asserts that the administration of cells can achieve diverse effects, including growth of any "hard" tissue or "soft" tissue (p. 20), formation of entire new organs (p. 32) or portions of organs (p. 46), restoration of function in any organ (p. 47), formation of auxiliary organs (p. 49), correction of necrosis (p. 49), replacement of missing limbs or body parts (p. 50), treatment of inflammation (p. 50), correction of musculoskeletal injuries or deficiencies (p. 50), formation of hybrid organs (p. 50), etc. No guidance or details are provided as to *how* to achieve these remarkable effects, most of which have never been achieved in this art to this day. The courts have stated that "[p]atent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable". Genentech Inc. v. Novo Nordisk A/S (CAFC) 42 USPQ2d 1001 (1997). The courts have also stated that "[t]ossing out the mere germ of an idea does

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not constitute an enabling disclosure... [R]easonable detail must be provided in order to enable members of the public to understand and carry out the invention" (Genentech Inc. v. Novo Nordisk A/S, supra).

3) The specification contains only prophetic examples. In fact, none of the prophetic examples are directed to administration of cells to grow a new artery, thus repairing a dead or damaged portion of a heart. Therefore, there are no examples, working or prophetic, directed to the elected invention.

4) The nature of the invention is highly complex, as evidenced by all of the publications of record, including Strauer. All inventions involving administration of active agents of any kind to a patient to achieve a physiological reaction are complex.

5) The state of the art does not support the specification's (and claims') assertion that a new artery can be grown. None of the numerous post-filing date publications put on the record by Applicant to support enablement of the claimed invention report the *de novo* growth of an artery as defined by Applicant, including Strauer.

6) The level of skill in the art is admittedly high.

7) The invention is unpredictable, as it involves administering active agents to a living patient to achieve a physiological response. As was found in Ex parte Hitzeman, 9 USPQ2d 1821 (BPAI 1987), most chemical reactions and physiological activity involve unpredictable factors. See also In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 927 F.2d 1200, 1212, 18 USPQ2d 1016, 1026 (Fed. Cir.), cert. denied, 502 U.S. 856 (1991).

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8) The breadth of the claims is quite large. The elected invention is directed to a method of administering any type of cell to an undefined area of a human body to grow new cardiac muscle and a new artery (of any type or location) to achieve growth of a new portion of a pre-existing heart.

Due to the large quantity of experimentation necessary to determine how to effectively administer cells to achieve *de novo* formation of cardiac muscle and an artery and thereby grow a new portion of a pre-existing heart, the lack of direction/guidance presented in the specification regarding the same, the absence of working examples directed to the same, the complex nature of the invention, the contradictory state of the prior art, the unpredictability of the effects of an agent on a physiological response, and the breadth of the claims which fail to recite limitations regarding cell type or dosage or site of delivery, etc., undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope.

Additionally, claims 248, 249, and 274-277 are directed to a method of administering cells to a human patient via *intravenous* or *intraluminal* injection to form new cardiac muscle and a new artery and cause growth of a new portion of a pre-existing heart (cl. 248, 249) and repair of dead/damaged portion of said heart (cl. 274-277). Such raises an additional enablement issue for the following reasons.

"Intravenous" is a term of art meaning administration into a vein. By definition, a vein is a blood vessel that leads toward the heart. "Intraluminal" is a term of art meaning administration into a "lumen" or cavity, such as the abdominal space or a blood vessel. It is noted that injection into the myocardium is an example of intramuscular

administration, not intraluminal administration. Intraluminal administration into a heart is when a substance is injected directly inside a chamber of the heart. The specification provides no detailed definitions of "intravenous" or "intraluminal" and thus the common, art-accepted definitions provided above are used herein to interpret the claims.

Again considering the guidelines set forth in In re Wands, *supra*, in the instant case, the quantity of experimentation required would be very large. The claims require administration of cells by intravenous or intraluminal injection to repair a dead or damaged portion of a heart. Administration of cells at a site distant to the site at which the cells are intended to adhere and grow had not been achieved in this art at the time of the invention. A great amount of experimentation would be required to determine how to administer the cells other than at the site of heart death/damage, cause the cells to travel to the site of heart death/damage, and then cause the cells to adhere such that repair of the dead/damaged heart portion could be achieved.

The amount of direction/guidance presented by the specification regarding these types of delivery is minimal. The words "intravenous" and "intraluminal" are used at p. 45 of the specification, and are restricted to the administration of VEGF **proteins**, not stem cells. The specification is silent with respect to overcoming the expected obstacles of targeting stem cells that are administered intravenously or intraluminally to the dead/damaged portion of the heart where they can adhere and exert their repairing effects. Thus, the skilled artisan is left with an invitation to experiment to determine how to administer cells intravenously or intraluminally as required by the claims.

There are no working examples directed to administering stem cells to dead or damaged portions of a heart. Although the specification contains prophetic statements that stems cells can be administered to a dead or damaged portion of a heart to repair the heart, no actual experiments or data were disclosed.

The nature of the invention is extremely complex. Evidence of this can be found in the relevant art. As stated in Murry et al. (of record, 1996, J. Clin. Invest. 98:2512-2523), "the goal of limiting myocardial injury has been difficult to achieve clinically, because ischemic myocardium dies quite rapidly and most patients wait more than 3 h after coronary occlusion before seeking medical attention" (p. 2512, Introduction).

The state of the prior art indicates that only localized injection of cells can successfully treat damaged myocardium. See Murry et al. (*supra*), Klug et al. (1996, J. Clin. Invest. 98:216-224), Oakley et al. (2001, Ann. Thorac. Surg. 71:1724-1733), Chiu et al. (1995, Ann. Thorac. Surg. 60:12-8), Yoon et al. (1995, Tex. Heart Inst. J. 22:119-125), Koh et al. (1993, J. Clin. Invest. 92:1548-1554), Van Meter et al. (1995, J. Thorac. Cardiovasc. Surg. 110:1442-1448), and Koh et al. (1995, J. Clin. Invest. 95:114-121). All used intramuscular injection of cells directly into the myocardium.

The level of skill of those in the art is admittedly high.

The art is considered unpredictable, since it could not be predicted if cells administered intravenously or intraluminally would reach the site of heart death/damage. Also, the courts have acknowledged that inventions utilizing biological systems are unpredictable. As was found in Ex parte Hitzeman, 9 USPQ2d 1821 (BPAI 1987), a single embodiment may provide broad enablement in cases involving predictable

factors such as mechanical or electrical elements, but more will be required in cases that involve unpredictable factors such as most chemical reactions and physiological activity. See also In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 927 F.2d 1200, 1212, 18 USPQ2d 1016, 1026 (Fed. Cir.), cert. denied, 502 U.S. 856 (1991). In the instant case, not even one single embodiment has been exemplified for this unpredictable system.

The claims are considered broad, since no details of the administration method other than "intravenous" or "intraluminal" are recited. For example, no dosages or targeting molecules are recited. No specific types of cells that would be expected to travel to the desired site are recited.

Due to the large quantity of experimentation necessary to determine how to administer cells intravenously or intraluminally to achieve growth of a new portion of a distant pre-existing heart, the lack of direction/guidance presented in the specification regarding the same, the absence of working examples directed to the same, the complex nature of the invention, the contradictory state of the prior art, the unpredictability of targeting cells to a distant site, and the breadth of the claims, it is determined that undue experimentation would have been required of the skilled artisan to practice the claimed methods.

Applicant's arguments (pp. 51-104, amendment received 26 June 2006) have been fully considered but are not found to be persuasive for the following reasons.

Applicant states that they understand the rejection to be based upon a lack of enablement for the administration of cells by intravenous and intraluminal techniques to

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a human patient to grow new cardiac muscle and a new artery and thus grow a new portion of a pre-existing heart. Applicant argues that the examiner incorrectly interpreted the claims as requiring repair of dead/damaged heart tissue. Applicant states that the USPTO is obligated to apply uniform standards of examination to maintain prosecution integrity and thereby ensure that administrative due process is accorded to all applicants. Applicant states that the examiner has applied inconsistent standards in concurrent examinations of the instant application and that of recently granted U.S. Patent 6,844,312 (Weiss). Applicant compares the instant application with the Weiss patent. Applicant's arguments have been fully considered but are not found to be persuasive. First, claims 274-277 now require repair of dead/damaged heart tissue. Next, each application is examined on its own merits, and the actions taken in the Weiss patent have no bearing on the instant procedure. Also, none of the claims in Weiss recite intravenous or intraluminal injection of cells. Furthermore, the therapeutic result required by the claims is different, the filing dates are different, the state of the art for each invention is different, and the disclosures are different. There has been no gross inconsistency on the part of the examiner.

Applicant reviews the legal standard for enablement with which the examiner takes no issue.

Applicant argues that the questioned intravenous and intraluminal administration techniques were well established in the medical arts prior to Applicant's invention. Applicant argues that cells, including stem cells, were well known and characterized prior to the invention. Applicant points to the existence of stem cell banks. Applicant

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argues that Dr. Elia's contribution to the medical arts was that an artery can be grown and a human heart repaired through use of a new combination of old administration techniques and old cellular materials. Applicant urges that one skilled in the medical arts would be enabled to make and use the claimed invention without resorting to more than routine experimentation based on the instant disclosure. Applicant also points to the expert opinions of Drs. Heuser and Lorincz as confirming this statement. This has been fully considered but is not found to be persuasive. The invention defined in claims 16, 17, 30, 31, and 47-52 is directed to a method of repairing a dead or damaged portion of a pre-existing heart comprising placing cells at a selected area of a human patient; and forming a new artery, thereby causing said dead or damaged portion of said heart to be repaired; wherein the cells are administered by intravenous or intraluminal injection. These claims thus require that the cells be administered at a location other than the site of the injury (e.g., the myocardium). The original rejection carefully considered all of the factors relevant to the question of enablement and whether or not undue experimentation would have been required of the skilled artisan to make and use the claimed invention. Please see pp. 4-8 of the non-final office action mailed 28 November 2003. Regarding the contributions of Dr. Elia to the art, such appears to be more relevant to the issue of novelty and obviousness than to the issue of enablement. The Heuser and Lorincz declarations will be addressed in turn.

Applicant states that the only evidence relied upon by the examiner is the Strauer et al. and Deb et al. publications. Applicant argues that the high-pressure, angioplasty balloon injection technique of Strauer was not a "specialized form of intraluminal

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delivery" as characterized be the examiner. Applicant argues that, whether or not the technique of Strauer was "specialized," it is allegedly evident that many other techniques may be used to perform the claimed method. Applicant refers to Wollert et al. (2004, Lancet 364:141-148) as achieving the required results using only a simple infusion of cells rather than a high pressure injection of cells. Applicant urges that, while the examiner has made an unsupported assertion to the contrary (in the advisory action at pp. 17-18), Wollert does not report high pressure injection and still achieves heart repair. Applicant concludes that the disclosure's failure to mention high pressure is of no moment because Wollert shows that lower pressure is operative. This has been fully considered but is not found to be persuasive. It is important not to lose sight of the fact that the claims recite intraluminal injection. This encompasses injection of cells into any lumen, which includes veins, arteries, intestines, intraperitoneal cavity, etc. Both Strauer and Wollert are limited to intraluminal injection into the infarct-related coronary artery, right at the site of the injured tissue. Thus, Strauer and Wollert do not constitute evidence to support enablement commensurate in scope with the claims. Regarding the high pressure injection question, Wollert discloses:

"6-8 h after bone-marrow harvest, the final preparation of bone-marrow cells was infused into the infarct-related artery via the central lumen of an over-the-wire balloon catheter (Concerto, Occam International Eindhoven, Netherlands). To allow bone-marrow cells maximum contact time with the microcirculation of the infarct-related artery, the balloon was inflated inside the stent to transiently interrupt antegrade blood flow during infusions. The entire bone-marrow cell preparation was infused during four to five coronary occlusions, each lasting 2.5-4 min. Between occlusions, the coronary artery was reperfused for 3 min." (third page of the electronic form of the document attached to the response to the final action, received 30 July 2004)

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It is respectfully submitted that, while Wollert does not actually use the words "high-pressure," that the method was actually high-pressure to achieve "interrupt antegrade blood flow" for "2.5-4 min." during "four to five coronary occlusions." High-pressure injection is necessarily achieved by a balloon catheter. The Wollert method is remarkably similar to the method used by Strauer:

"Five to nine days after the onset of acute infarction, cells were directly transplanted into the infarcted zone (Figure 1). This was accomplished with the use of a balloon catheter, which was placed within the infarct-related artery. **After exact positioning of the balloon at the site of the former infarct-vessel occlusion, percutaneous transluminal coronary angioplasty (PTCA) was performed 6 to 7 times for 2 to 4 minutes each.** During this time, intracoronary cell transplantation via the balloon catheter was performed, using 6 to 7 high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells. **PTCA thoroughly prevented the backflow of cells...**" (p. 1914, emphasis added).

In conclusion, both Strauer and Wollert use remarkably similar techniques to implant cells at the site of the infarct-related injury. Such does not constitute evidence commensurate in scope with the rejected claims, which merely recite intravenous or intraluminal injection of cells. Finally, It is noted that the instant specification only states that cells ("multifactorial and non-specific cells" or "stem cells" or "germinal cells") can be used to grow an organ or repair/replace dead or damaged heart tissue. No specific guidance regarding *how* to obtain appropriate cells, *how* to administer them, *how* to monitor success, etc. are provided. The Strauer and Wollert references provide evidence of the large quantity of experimentation that was still required after Applicant's claimed priority date in order to achieve some beneficial result.

Applicant argues that Strauer 2002 did not state that other administration techniques, such as intramuscular or intravenous, were inoperative but instead considered such techniques not as efficient as his technique. Applicant refers to Strauer 2003 as supporting intravenous administration as "easiest." Applicant refers to Phase I trials of Osiris as supporting enablement of intravenous administration of cells for heart repair. This has been fully considered but is not found to be persuasive. Strauer 2003 does not report any results after using intravenous administration, and cannot be interpreted as indicating that intravenous administration is easiest. Furthermore, the diagram in Strauer 2003 shows intravenous administration into a vein on the surface of the heart, not at a site distant from the heart. Osiris also does not report efficacy results. No experimental details are provided for either Strauer 2003 or Osiris, so it is impossible to determine if the evidence is commensurate in scope with the claims or if different methodologies or pharmaceuticals were used.

Applicant argues that Strauer 2002 does not provide a side-by-side comparison, and thus the examiner's comments are speculative. Applicant refers to cf. Hormone Research Foundation v. Genentech, Inc., 904 F.2d 1558, 15 USPQ 2d 1039 (Fed.Cir.1990). Applicant argues that all an applicant is required to do is to provide a disclosure that one skilled in the art can understand and then follow to make and use the invention. Applicant concludes that any failure to disclose a later developed technique has no bearing upon enablement. This has been fully considered but is not found to be persuasive. Strauer 2002 clearly indicates that intravenous administration is not expected to provide beneficial results. See p. 1917, left column, second

paragraph. The issue is not whether or not Applicant is required to foresee improvements, but whether or not sufficient guidance is present in the specification as originally filed to enable one skilled in the art to make and use the claimed invention without resorting to undue experimentation. The fact that the references published well after the claimed priority date reported the necessary development of techniques and materials to successfully achieve repair of damaged myocardium, wherein these techniques and materials are not disclosed in the instant specification, evidences the significant amounts of further experimentation that was required to achieve growth of a new portion of a pre-existing heart by administration of cells. Such development of new techniques and materials constitutes part of the act of invention. Regarding Hormone Research Foundation v. Genentech. Inc., 904 F.2d 1558, 15 USPQ 2d 1039 (Fed.Cir.1990), the court found that "[t]he '833 specification itself discloses that Dr. Li's claimed method had produced the material depicted in Figure 1 of the '833 patent and that such a material exhibited lactogenic activity. Evidence tending to support this assertion can be found in several of the journal articles of record." Thus, the disclosure at issue in Hormone Research Foundation v. Genentech. Inc. disclosed considerably more detailed disclosure compared to the instant application, included working examples and figures, and was supported by other published evidence. The instant fact pattern is more akin to that in Genentech Inc. v. Novo Nordisk A/S (CAFC) 42 USPQ2d 1001 (1997). The court stated that "[p]atent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable". The court also stated that "[t]ossing out the mere germ of an

idea does not constitute an enabling disclosure... [R]easonable detail must be provided in order to enable members of the public to understand and carry out the invention" (Genentech Inc. v. Novo Nordisk A/S, *supra*).

Applicant again refers to the Weiss patent. The Weiss patent is not relevant to the issues of the instant application, since each application is examined on its own merits.

Applicant argues that the examiner raised generalized concerns regarding properties and handling of cells in the prosecution of the instant application. Applicant characterizes these concerns as opinion rather than factual evidence. Applicant argues that properties of cellular materials were well established prior to the filing date and refers to several documents in support of such. Applicant argues that official notice can be taken that stem cell culture techniques have been known and used decades prior to Applicant's filing date, and that any skilled person in the medical arts would be familiar with the properties and stem cell handling techniques at issue. This has been fully considered but is not found to be persuasive. The rejection was based on evidence (see publications cited at p. 7 of the non-final office action mailed 28 November 2003, as well as Strauer and Wollert) and sound scientific reasoning, not mere opinion. Proper legal analysis of all of the Wands factors was set forth on the record (non-final office action mailed 28 November 2003). The first of four pieces of evidence referred to by Applicant, the Caplan abstract, does not overcome the rejection. Caplan discusses the differentiation of cells *in vitro* into specialized cells for localized administration, and the use of cells as gene therapy vectors. Such is not relevant to the issue at hand. The

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second piece of evidence, Merck has to do with cancer and does not appear to be relevant. The third piece of evidence, NIH report, lists results of a web search for "nonspecific growth factor" and also appears to be irrelevant to the issue at hand. The last piece of evidence, Exhibit III in the after final amendment, reviews traditional use of cells for cancers and immunotherapy, and newer uses as gene therapy vehicles. None of these treatments involve the systemic administration of cells to repair a distant organ.

Applicant argues that administration of cells is old in the art. This point is conceded.

Applicant argues that the examiner's statement regarding Deb et al., wherein it was acknowledged that cells administered intravenously could migrate to the art, should end all speculation regarding enablement of the claimed invention. This has been fully considered but is not found to be persuasive because Deb et al. do not demonstrate that cells can migrate to the heart in sufficient quantities to repair any defects. Deb discloses that only $0.23 \pm 0.06\%$ of the cardiomyocytes were from the transplanted cells. Such numbers of cells are greatly insufficient to achieve the effects required by the claims. As evidence of this, Strauer 2002 administered 6 to 7 fractional high-pressure infusions of 2 to 3 mL cell suspension, each of which contained 1.5 to 4×10^6 mononuclear cells directly to the infarct site in order to achieve their effects. In fact, Strauer 2002 specifically points to shortcomings of intravenous administration at p. 1917. The evidence as a whole indicates that intravenous administration of cells to repair a dead or damaged portion of a heart has not yet been achieved due to the obstacles involved with getting sufficient numbers of cells to the dead/damaged site and

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preventing them from re-migrating away from the site. As this problem has not yet been solved in the literature, and no suggestions for solving the problem are suggested in the specification as originally filed, undue experimentation would be required of the skilled artisan to practice the claimed method to achieve the required result.

Applicant argues that Deb et al. specifically suggest that human bone marrow can be used as a source of extracardiac progenitor cells capable of de novo cardiomyocyte formation at p. 2 of the conclusion section. This has been fully considered but is not found to be persuasive because Deb et al. do not suggest administration of bone marrow cells intravenously to achieve cardiomyocyte formation. Rather, they suggest that bone marrow can be used as a *source*.

Applicant argues that Strauer 2002 and Wollert also provide evidence that cells can migrate to the infarct zone. This has been fully considered but is not found to be persuasive. Strauer 2002 and Wollert administered cells at the heart. Such is not commensurate in scope with "intravenous" or "intraluminal" administration, which reads on administration at sites far distant from the heart (e.g., a vein in the arm, the lumen of the intestinal tract).

Applicant argues that the lack of teachings regarding dosages in Deb et al. is irrelevant since Deb et al. were not addressing repair of damaged heart tissue. Applicant argues that it would have been routine to administer cells multiple times to achieve the desired result. Applicant points to Strauer 2002 as not teaching that intravenous administration is inoperative. Applicant points to Strauer as using seven infusions, and p. 45 of the specification as suggesting sequential administrations of

growth factors. This has been fully considered but is not found to be persuasive. Deb et al. Page 45, lines 26-27 of the specification read as follows :

"It may be necessary to provide gene(s) or growth factor(s) sequentially. For instance, one or more blood vessels are grown by inserting an appropriate gene or other genetic material into a selected area. Second, an appropriate gene or other genetic material is inserted in the selected area to grow a bone or other organ." Clearly, this section of the specification is directed to sequential administrations to achieve *different* effects. There is no guidance regarding multiple administrations to achieve one effect. Also, there is no guidance in the specification or the prior art regarding how many intravenous or intraluminal administrations of cells are needed to achieve growth of a new portion of a pre-existing heart. Deb et al., Strauer 2002, and Wollert cannot be relied upon to provide the missing guidance, since they were published after the instant filing date.

Applicant argues Applicant argues that coupling Deb and Strauer is inappropriate since Deb is not concerned with growth of a new portion of a pre-existing heart. Applicant urges that the examiners' position is without evidence. Applicant argues that Strauer indicates that intravenous administration is operative. Applicant concludes that it was well within the skill of the art to select an appropriate number of cells and number of infusions. This has been fully considered but is not found to be persuasive. Since Applicant has relied on both Deb and Strauer, it was not inappropriate to point out deficiencies in the references. Strauer 2002 clearly teaches away from intravenous

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administration. Finally, the concept of multiple administrations for a single effect does not appear to be disclosed in this specification.

Applicant argues that "remigration" is not an insurmountable problem because Wollert succeeded. Applicant argues that the examiner has fabricated problems and that the invention need not be optimized in order to be patentable. This has been fully considered but is not found to be persuasive. Again, it is important to remember that Wollert is limited to intraluminal injection into the infarct-related coronary artery, right at the site of the injured tissue. Thus, Wollert does not constitute evidence to support enablement commensurate in scope with the claims, which encompass intravenous or intraluminal administration at any vein or lumen. Furthermore, the specification does not provide guidance along the lines of Wollert's use of large quantities of cells and multiple administration passages to overcome re-migration problems identified by others in this art.

Applicant provides comments regarding the multiple declarations by Drs. Heuser and Lorincz. Applicant urges that Drs. Heuser and Lorincz are eminently qualified, and that the examiner has improperly dismissed the evidence of the declarations. Applicant argues that enablement is a question of fact. Applicant argues that the evidence indicates that only routine experimentation would have been required of the skilled artisan to make and use the claimed invention. This has been fully considered but is not found to be persuasive. There is no question that Drs. Heuser and Lorincz are distinguished doctors. However, in assessing the weight to be given expert testimony, the examiner may properly consider, among other things, the nature of the fact sought

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to be established, the strength of any opposing evidence, the interest of the expert in the outcome of the case, and the presence or absence of factual support for the expert's opinion. See Ex parte Simpson, 61 USPQ2d 1009 (BPAI 2001), Cf. Redac Int'l. Ltd. v. Lotus Development Corp., 81 F.3d 1576, 38 USPQ2d 1665 (Fed. Cir. 1996), Paragon Podiatry Lab., Inc. v. KLM Lab., Inc., 948 F.2d 1182, 25 USPQ2d 1561, (Fed. Cir. 1993). The nature of the fact sought to be established is whether or not more than routine experimentation would have been required to practice the claimed invention in its full scope. This issue has been extensively addressed on the record with reference to the Wands factors and the publications of record. It is maintained that more than routine experimentation would have been required. The strength of opposing evidence has also been addressed extensively on the record. The post-filing date publications are filled with specific guidance necessary to achieve the desired results. This specific guidance is absent in the instant specification. Finally, the claims are incredibly broad, reciting general intravenous or intraluminal administration. The post-filing date art that achieves any growth of new portions of pre-existing hearts used specific administration methods that are not specifically pointed to in the specification. Regarding the interest of the experts in the outcome of the case, there is no evidence that there is any such interest. Finally, there is a question of the presence or absence of factual support for the expert's opinion. Mostly, the experts relied upon the specification itself, which has been separately addressed. However, some publications were also referred to. These have been addressed on the record. Thus, the declarations have been fully considered and a finding that the rejection should be maintained is proper. As an aside, it is

respectfully submitted that Applicant is mistaken in their statement that enablement is a question of fact. Case law has established that anticipation and operativeness are questions of fact; however, obviousness and enablement are questions of law. See In re Lindell, 155 USPQ 521; In re Chilowsky, 134 USPQ 515. Thus, while no weight is given to the experts' opinion regarding the ultimate legal conclusion of enablement, the underlying basis for the legal conclusion has been considered.

Applicant next discusses the Wands factors. Again, the Wands factors have been extensively reviewed on the record. Applicant's arguments are duplicative of arguments already made and addressed on the record. In view of consideration of the preponderance of the totality of the evidence, the rejection is maintained.

35 U.S.C. § 112, Second Paragraph

Claim 245 remains rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Applicant's arguments (pp. 36-41, amendment received 26 June 2006) have been fully considered but are not found to be persuasive for the following reasons.

Applicant argues that the rejection is inconsistent with the decision on a parent patent, 5,759,033. This has been fully considered but is not found to be persuasive. The prior examiner's position is not binding. Furthermore, in general, it is not the policy of the USPTO to perpetuate errors. When issues are first identified, they must be raised.

Applicant refers to the Philips decision. The same argument appeared in the response received 21 November 2005 and has already been addressed in the office action mailed 16 February 2006.

Applicant refers to the declarations of Drs. Heuser and Lorincz. These have also already been addressed.

Applicant refers to Exhibits as additional evidence in support of their position. While these references do, in fact, refer to proteins as multifactorial, they define the exact, specific effects the proteins have. Therefore, the entire phrase "multifactorial and non-specific" as it relates to cells, is still not defined.

Applicant points to Strauer 2005, Caplan 1991, and Caplan 2001 as using the term "multifactorial" to describe cells. Strauer 2005 does not use the term multifactorial to describe cells. Rather, Strauer 2005 uses "four mechanisms" to describe "regenerative potential" and not cells *per se*. Also, Strauer 2005 only discusses bone marrow cells, which are already indicated by the specification as exemplary of "multifactorial and non-specific" cells, and thus does not provide evidence regarding what cells other than stem cells and germinal cells can be termed "multifactorial and non-specific." Regarding Caplan 1991, the examiner is at a loss as to how Applicant can conclude that the publication uses the term "multifactorial and non-specific" to describe MSCs from the quoted passages. Caplan 2001 uses the term "multifactorial" to describe the differentiation pathway, a process, and thus supports the examiner's position.

Applicant refers to Exhibit G. This describes a drug as a non-specific growth factor for megakaryocytes. This also does not resolve the issue since it does not address the question of what "multifactorial and non-specific" means in terms of cells.

Applicant argues that the phrase "multifactorial and non-specific" has been used to describe or characterize the potentialities of stem, germinal, and pluripotent cells. This has been fully considered but is not found to be persuasive. While the phrase "multifactorial and non-specific cells" appears in the specification, there is no clear definition of what cell types are encompassed by the term, for the reasons of record. Page 37 of the specification states, "Multifactorial and nonspecific cells (**such as** stem cells and germinal cells) can provide the necessary in vivo and in vitro cascade of genetic material once an implanted master control gene's transcription has been activated" (emphasis added). The use of "such as" clearly implies that the term "multifactorial and non-specific cells" is intended to encompass cells other than stem cells and germinal cells. However, neither the specification nor the art disclose what these other cells are. In the absence of this information, the skilled artisan cannot determine the metes and bounds of the claims at issue.

In conclusion, the term "multifactorial and non-specific cells," recited in claim 245, is not defined unambiguously in the art or in the specification, for the reasons set forth above. Therefore, the skilled artisan cannot determine the metes and bounds of the claimed invention, and the rejection is proper.

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Double Patenting

Claims 286 and 287 of this application conflict with the claims of Application No. 09/794,456. 37 CFR 1.78(b) provides that when two or more applications filed by the same applicant contain conflicting claims, elimination of such claims from all but one application may be required in the absence of good and sufficient reason for their retention during pendency in more than one application. Applicant is required to either cancel the conflicting claims from all but one application or maintain a clear line of demarcation between the applications. See MPEP § 822.

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 286 and 287 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over the claims of copending Application No. 09/794,456. Although the conflicting claims are not identical, they are

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not patentably distinct from each other because the instant and c-pending claims are generally directed to methods of repairing dead or damaged portions of a pre-existing heart by administering stem cells and forming a new artery.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Conclusion

No claims are allowed.

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

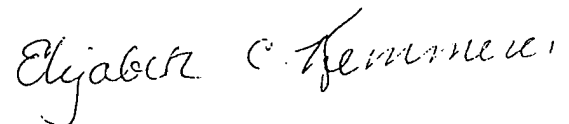
A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

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Any inquiry concerning this communication or earlier communications from the examiner should be directed to Elizabeth C. Kemmerer, Ph.D. whose telephone number is (571) 272-0874. The examiner can normally be reached on Monday through Thursday, 7:00 a.m. to 5:30 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Nickol, Ph.D. can be reached on (571) 272-0835. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.



ECK

ELIZABETH KEMMERER
PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 09/836,750	Applicant(s)/Patent Under Reexamination ELIA, JAMES P.	
	Examiner Elizabeth C. Kemmerer, Ph.D.	Art Unit 1646	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
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of Genetics and Cytogenetics

Classical and Molecular

Fourth Completely Revised Edition

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4. Crossing-over frequencies are the values needed for exact mapping. In contrast to recombination values, they are always strictly additive: the sum of crossing-over frequencies for two adjacent regions (e.g., a-b and b-c) is equal to the frequency of crossing-over in the whole region (a-c). The crossing-over frequency can be obtained from the observed recombination frequency by correcting for the effects of multiple (double) crossing-over and \rightarrow interference. Because of interference the correction is mathematically rather complex. The unit measure of chromosome length and distance between any two markers is the \rightarrow "map unit" (\rightarrow "crossing-over unit" or Morgan unit) which is equal to the corrected recombination frequency, expressed as a percentage. One map unit is defined as the corrected recombination frequency of 1 per cent or one centimorgan. The relative distance between two markers calculated from uncorrected recombination frequencies (the recombination value) is referred to as their "apparent distance", as opposed to their "actual distance", i.e. the distance calculated from the recombination frequency corrected for multiple crossing-over (the "exchange value"). An estimate of actual distances between genes follows from the construction of cytological maps (\rightarrow chromosome map) or their equivalents. These are made independently of recombination data.

Mapping according to these principles represents the genes as seemingly dimensionless points on the g. m. The number of offspring tested for recombination is of the order of $10^2 - 10^3$. Under these conditions, it is unlikely that rare recombinations will be discovered. In systems allowing the study of $10^6 - 10^7$ progeny, unique types of (rare) recombinants are recovered as a result of crossing-over within the limits of single genes (intragenic crossing-over). In such systems, the genetic resolving power ("the genetic resolution power") is greatly increased. The locus of a gene ceases to appear as a dimensionless point; by "genetic fine structure analysis" via recombination tests of high resolving power (\rightarrow deletion mapping), the gene is shown to be composed of a linear sequence of recombining mutational sites (\rightarrow gene mutation) whose positions can be mapped. (\rightarrow allele; genetic code).

In systems where for one reason or another recombination-type mapping cannot be effectively employed, some alternative approaches may be used. DNA replication polymerase and transcription polymerase offer an alternative basis for g. mapping. (\rightarrow replicational mapping; transcriptional mapping; heteroduplex mapping; temporal mapping).

genetic mapping — any method used in the measurement of positions and relative distances between genes of a \rightarrow linkage group or sites within a gene (fine scale mapping). (\rightarrow genetic map).

prevent the growth of parental genotypes, manifesting a particular phenotype (e.g., growth factor auxotrophy), in particular environments (media), whereas recombinants for these markers are able to grow freely in the same environment, i.e., have a selective advantage over the parental types, thus facilitating their quick identification.

Unselected markers are without selective advantage for the recombinants as compared to the parental genotypes. Here the identification of recombination is determined primarily by the internal recombination mechanism and less by the technique applied. Under these conditions segregation is determined as a second step procedure.

In principle, the same markers may be used as selected or unselected ones dependent on the experimental conditions used.

Genetic material — the carrier of primary \rightarrow genetic information: single or double-stranded \rightarrow deoxyribonucleic acid (single in some, double in most bacteriophages, bacteria and higher organisms) or \rightarrow ribonucleic acid (in RNA-viruses). G. m. must fulfil at least two fundamental functions: 1. serve as a template for its own \rightarrow replication ("autocatalytic function"); 2. provide a template for the synthesis of other classes of macromolecules (specifically proteins), i.e., supply the structural and regulatory information it contains to the protein-synthesizing machinery of the cell ("heterocatalytic function").

Genetic message — a \rightarrow messenger RNA molecule coding for a single, functional polypeptide. A \rightarrow polycistronic mRNA carries several messages.

Genetic mimic — \rightarrow genocopy.

Genetic mobility (Darlington 1958) — the ability of biological species to change their habitat or (in plants) the range of distribution of pollen and seeds. It is expressed differently in different individuals. The g. m. largely determines the manner whereby the mechanism of geographic \rightarrow isolation becomes effective.

Genetic mosaic — any individual (called \rightarrow chimera or mosaic) which is composed of genetically different tissues and displays distinct characters in different sectors of the individual. Mosaic areas may correspond to the expression of different alleles contained in the particular genotype, or may arise by \rightarrow gene mutation, \rightarrow chromosome mutation, somatic crossing-over and somatic segregation, aneuploidy, or double \rightarrow fertilization. Genetic mosaics are called \rightarrow gynandromorphs or gynanders if part of the individual is male, part is female.

Genetic nomenclature — the designation of genes by symbols. Mendel (1865) was the first to use capitals (e.g. A) to represent dominant and small letters (e.g. a) to represent recessive characters without using letters specifically derived from the particular type of expression of the character pair under observation.

Today, genes (and characters) are designated by the letters of the Roman alphabet or by abbreviations of the designations (in Latin or English) given by the person who discovered the phenotypic character pair controlled by the gene pair in question. Generally, the recessive allele of a gene pair is marked by a small letter, the corresponding dominant allele by the same letter or letters, but beginning with a capital. Another

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